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Award Number: DAMD17-01-1-0209

TITLE: Analysis of Apaf-1 and Caspase 9 in Tumorigenesis

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REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2004	3. REPORT TYPE AND DATES COVERED Annual Summary(1 Jul 2001 - 30 Jun 2004)	
4. TITLE AND SUBTITLE Analysis of Apaf-1 and Caspase 9 in Tumorigenesis		5. FUNDING NUMBERS DAMD17-01-1-0209	
6. AUTHOR(S) Masashi Narita, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724		8. PERFORMING ORGANIZATION REPORT NUMBER	
E-Mail: narita@cshl.edu			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		20041028 107	
11. SUPPLEMENTARY NOTES Original contains color plates: ALL DTIC reproductions will be in black and white			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The replicative exhaustion of Human diploid fibroblasts (HDFs) in culture is associated with an "irreversible" cell-cycle arrest known as replicative senescence. However, young cells can enter a similar phenotype to replicative senescence in response to expression of certain mitogenic oncogenes, oxidative stress, and DNA damage. Cellular senescence involves activation of the p53 and p16/Rb tumor suppressor pathways. We identified a novel type of chromatin structure that has features of heterochromatin and appears to contribute to the senescence state. We designated these chromatin structures as SAHFs, <u>Senescence Associated Heterochromatic Foci</u> . SAHF formation is dependent on the p16/Rb pathway, which is often abrogated in breast cancer. We also showed that some cell-cycle genes are stably silenced in senescence. Here we further characterized SAHFs and found the distinct localization polycomb (some P ^c G proteins are implicated in p16 regulation) in SAHFs. Moreover, we found that enforced expression of SUV39H1 (a histone H3 lysine 9 methyltransferase; HMT), which can form complex with Rb, induced senescence like phenotype in HDFs, which underscores importance of chromatin modifications in senescence. We propose that Rb-dependent changes in chromatin organization are critical in senescence and some HMT and P ^c G might be involved in SAHF formation.			
14. SUBJECT TERMS senescence, p53, Rb, p16		15. NUMBER OF PAGES 49	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	10
References.....	17
Bibliography of all publications.....	18
Appendices.....	19
Meeting abstracts.....	19
Curriculum Vitae.....	23
Attached Papers.....	25

Introduction

The ultimate goal of this project is to uncover the molecular mechanisms of tumor suppression in breast cancer, which include apoptosis and cellular senescence mechanisms. The objectives outlined in our original proposal were designed to elucidate the mechanism of the tumor suppression through the p53-dependent apoptosis pathway, using a genetic approach. As already mentioned in the previous annual reports and the New Statement of Work, we shifted the focus of the research from apoptosis to senescence. Senescence appears to involve the Rb and p53 tumor suppressor pathways. Hence, as cells enter a senescent state, p53 and p16 accumulate, and Rb becomes hypophosphorylated. In breast cancer, approximately 50 % of tumors show low or lack of p16 expression, and many have mutations in Rb or p53. It is also significant that cells derived from mammary tissues almost always lose p16 expression when cultured *in vitro* (1). These observations suggest that mammary cells are more likely to inactivate the senescence pathway by losing p16 expression. Recently we found that the p16/Rb pathway is also critical for formation of Senescence Associated Heterochromatic Foci (SAHFs). (2) (Narita, attached paper) We propose that SAHF formation, which involves the stable silencing of cell cycle genes, confers an 'irreversible' growth arrest.

Body of Annual Report

Senescence was initially identified as a permanent form of cell cycle arrest that accompanied the replicative exhaustion of human fibroblasts in culture. However, recent work from our laboratory and elsewhere has shown that senescence is induced by many forms of cellular stress. By analogy to apoptosis, we have been seeking the “common machinery” of cellular senescence. A large body of evidence indicates that both the p53 and Rb tumor suppressor pathways contribute to cellular senescence. Our laboratory and others reported that p53 is required for the induction of ‘premature’ senescence by oncogenic ras (3, 4, 5). However, p53 is not essential for maintaining senescence (6). We recently reported that senescent human fibroblasts form senescence associated heterochromatic foci (SAHFs), and that the formation of these structures was dependent on the presence of an intact p16/Rb pathway (2) (Narita, attached paper). Interestingly, Beausejour et al successfully reversed the senescent phenotype in BJ cells. These cells do not form SAHFs and p16 is not up-regulated. In contrast, they could not reverse senescence in the other type of human diploid fibroblasts (HDFs), WI38 cells, which do form prominent SAHFs and do up-regulate p16. These observations further underscore the correlation between SAHF formation and the stability of the senescence phenotype (7) (Beausejour, attached paper).

A. Molecular mechanism of SAHF formation (Aim 1)

As we described in the previous annual reports, senescent HDFs form characteristic heterochromatic structures, SAHFs (2) (Narita, attached paper). SAHFs are novel nuclear structures that may ultimately provide clues into senescence biology. Therefore, we decided to extend the characterization of SAHFs and present our recent findings below.

H3 K9triM/HP1 and H3 K27/Pc localize in a distinct pattern in SAHFs

There are several lines of evidence, which suggest that epigenetic regulation of gene expression is involved in the senescent state (8). Previous studies indicated that H3 lysine 9 methyl (K9M) preferentially associates with HP1 whereas H3 lysine 27 methyl (K27M) preferentially associates with polycomb (Pc), and both complexes have been implicated in senescence (9). In addition, specific histone lysines can be either mono-, di-, or tri- methylated, and the extent of methylation has an influence on the transcription and chromatin status. In the previous annual report, we have shown that, H3 K9triM and HP1 γ were strongly enriched in the core region of SAHFs, while H3 K9diM stained the whole area of SAHFs. Here, we examined the H3 K27M pattern in SAHFs using an H3 K27M antibody from Dr. Danny Reinberg (University of Medicine and Dentistry of New Jersey). Strikingly, H3 K27M is enriched in the region, surrounding the H3 K9triM/HP1 core region as shown in Figure 1. To identify the K27M binding partner, we tested the available antibodies against polycomb group (PcG) proteins, including Human Pc2, EZH2, Bmi1 (gift from Dr. Otte). Surprisingly, none of them showed the K27M pattern in SAHFs (data not shown). Next, we generated GFP-fusion constructs to other PcG proteins, such as M33 and EED. Since complete sequence of human Pc1 cDNA has not been identified, we used mouse Pc1 (M33). M33 colocalizes with H3 K27M, while a PcG protein EED did not (Figure 2A and data not shown), suggesting that human Pc1 might be the binding partner for the K27M in SAHFs. We are presently cloning the putative human Pc1 cDNA in collaboration with Dr. Beach’s lab. We are also generating specific RNAi constructs for the putative human Pc1 to explore the significance of the H3 K27M/Pc1 complex in SAHFs (see model; Figure 2B).

B. Analysis of the chromatin status of specific genes in senescent cells (Aim 2)

Using two different H3 K9diM antibodies (both of which cross-react with H3 K9triM and

K27triM), we have shown that some E2F-targets genes are in the heterochromatin fraction in senescent cells (2) (Narita, attached paper). To more specifically examine the H3 modification by ChIP analysis, we needed to generate highly specific, high titer antibodies. First we tried to generate a rabbit polyclonal antibody against H3 K9triM (previous report). Sera from those rabbits also reacted with H3 K9diM, K9triM, and K27triM. We successfully purified the K9triM specific fraction from those sera for immunostaining assay. However, the yield of this fraction was very low. We tried to produce a higher titer antibody but the specificity of the sera decreased during the course of immunization of the rabbits. We next focused on generating monoclonal antibodies against H3 K9diM and K9triM. To date, we established three hybridoma cell lines for H3 K9diM and K9triM (Table 1). Unfortunately, we could not generate the K9triM specific monoclonal antibodies; they cross-react with K9diM or K27triM. We are now in the process of large-scale purification of these monoclonal antibodies for ChIP analysis.

C. Analysis of senescent phenotype in mammary epithelial cells (Aim 3)

We have shown that SAHF-formation is partially dependent on p16 expression using a p16 specific short hairpin RNA (2). Also, we observed that different cell types have different induction of p16 upon senescence. For example, BJ cells (human skin fibroblasts) show a minimal p16 up-regulation and SAHF formation, while senescent IMR90 and WI38 cells (human embryonic lung fibroblasts) show prominent SAHFs and p16 induction, indicating the correlation between p16 level and SAHF formation. Therefore, we asked if mammary epithelial cell lines, which typically lack p16, can be induced to senesce by p16 expression. We used a near diploid non-tumorigenic breast epithelial cell line (MCF10), which lacks p16 expression. Upon p16 expression, cells showed senescence-like features, such as SA- β -galactosidase activity, typical senescence morphology, and cell cycle arrest. However, SAHFs did not form, suggesting that these cells may lack some component, which is required for SAHF-formation. This indicates that mammary cell may be deficient in maintaining a senescent state stably.

D. Role of HP1 α and β in tumor suppression *in vivo* (Aim 4)

The putative role of HP1 proteins in maintaining senescence is consistent with a tumor suppressor activity. Consistent with this view, HP1 α is not expressed in a subset of breast cancers (10). To test this, we have obtained ES cells that have insertional mutations in the HP1 α genes (gene trap method) from Dr. Harald Von Melchner (University of Frankfurt). The HP1 α +- ES cells have produced chimeric mice. The HP1 β +- cells have gone germline (University of Frankfurt). Unfortunately, none of HP1 α +- ES cells underwent germline transmission here in our facility. Therefore, a different series of HP1 α +- ES cells were injected in the University of Frankfurt, and have undergone germline transmission successfully. We are in the process of obtaining those mice. And then we will generate HP1 α and β double knockout mice.

E. SUV39H1 overexpression induces some features of senescence.

Our results suggest that H3 K9M is important for stabilizing the senescent state (2) (Narita, attached paper). Histone methylation requires the action of histone methyltransferases (HMTs). SUV39H1 is the major HMT to H3 K9triM. SUV39H1 can also form complexes with HP1 and Rb (11). To determine whether SUV39H1 can affect senescence, we cloned human SUV39H1 cDNA by RT-PCR using total RNA isolated from IMR90 cells, and overexpressed SUV39H1 in IMR90 cells. Remarkably, enforced expression of SUV39H1 induces cell cycle arrest, senescence morphology, and SA- β -gal activity in a manner that is dependent on its SET domain (the catalytic site) (Figure 3). However, SUV39H1-induced senescence phenotype was not accompanied by SAHFs (~ 10%, data not shown). Furthermore, SUV39H1 associates poorly with chromatin in senescent cells

(Figure 4A, Ras lane), raising the possibility that a different HMT may be playing a major role. Currently, we are attempting to identify key HMTs in senescence using retroviral RNAi (short hairpin RNA) technology against a series of HMTs in collaboration with Dr. Greg Hannon (Cold Spring Harbor Lab.) (Table 2). We designed a short hairpin against SUV39H1 that specifically knocks down SUV39H1 levels (Figure 5) and are characterizing its effect on senescence.

Key Accomplishments

- Distinct pattern between H3 K9triM/HP1 and H3 K27/Pc in the SAHF.
- M33 colocalizes with H3 K27M in SAHF.
- Establishment of three new monoclonal hybridoma cell lines against Histone H3 Lys9 methylation.
- SUV39H1 induces senescence-like phenotype in IMR90 cells.
- Generation of efficient short hairpin against SUV39H1.

Reportable Outcomes

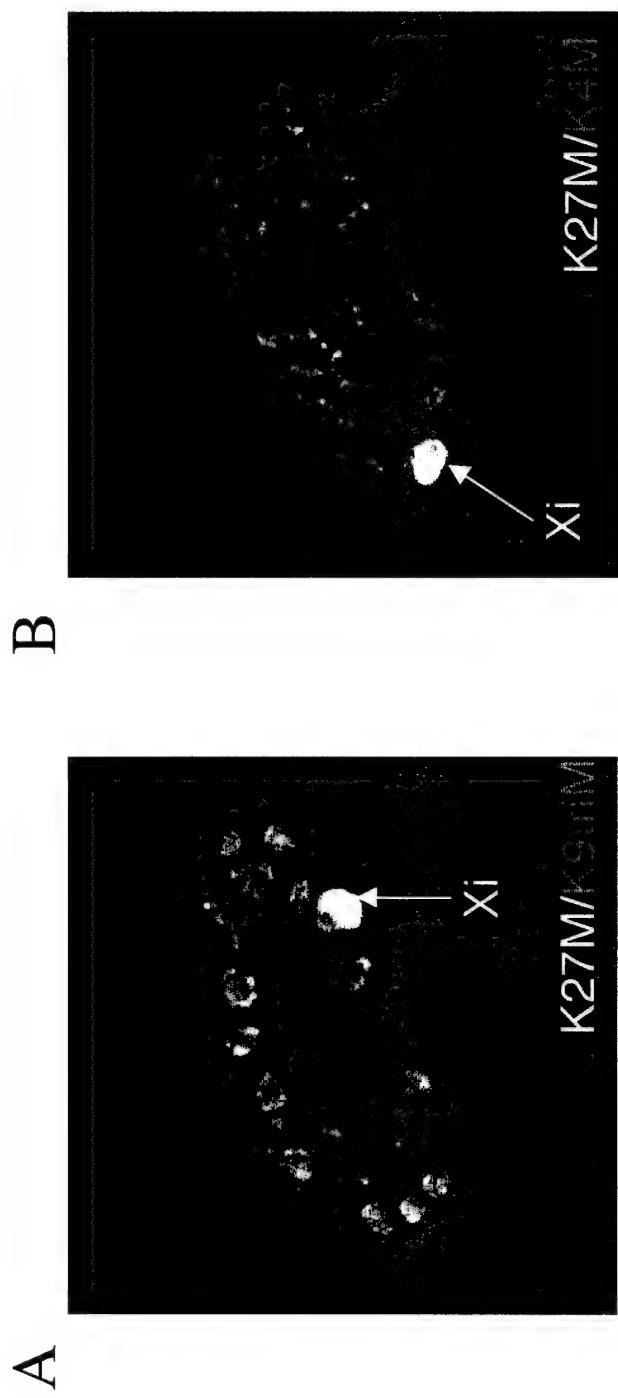
Narita M, Lowe SW. Executing Cell Senescence. *Cell Cycle*, 2004 Mar;3(3):244-246

Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, Campisi J. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.*, 2003 Aug 15;22(16):4212-22

Conclusions

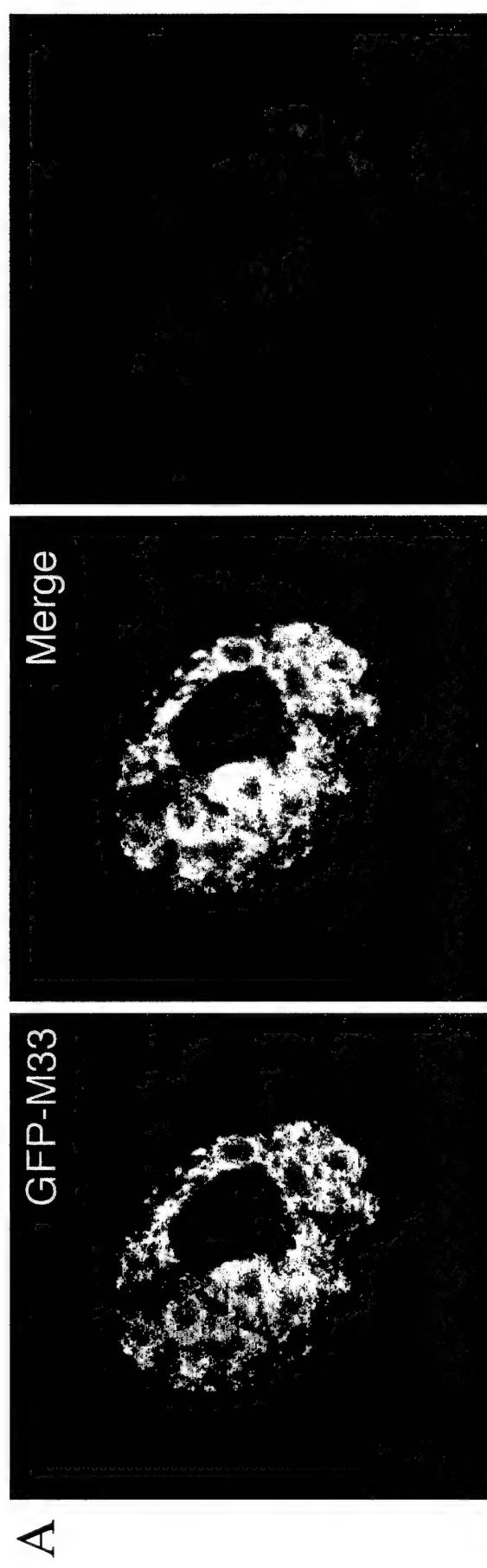
We have identified the novel type of heterochromatic structure (SAHFs) that is associated with senescent phenotype in human fibroblasts. We further characterized SAHFs and found a distinct localization of histone H3 K9triM and H3 K27M. Furthermore, while HP1 colocalizes with H3 K9M, we found that the ectopic mouse Pcl (M33) was enriched in H3 K27M region in SAHFs, indicating that H3 K27M/Pcl complex may be involved in SAHF-formation. We also found that SUV39H1, a key enzyme for H3 K9triM, induces senescence in HDFs. Our continuing studies will provide insights into the involvement of heterochromatic proteins and Pcg proteins in senescence and tumor suppressor functions.

Figure 1



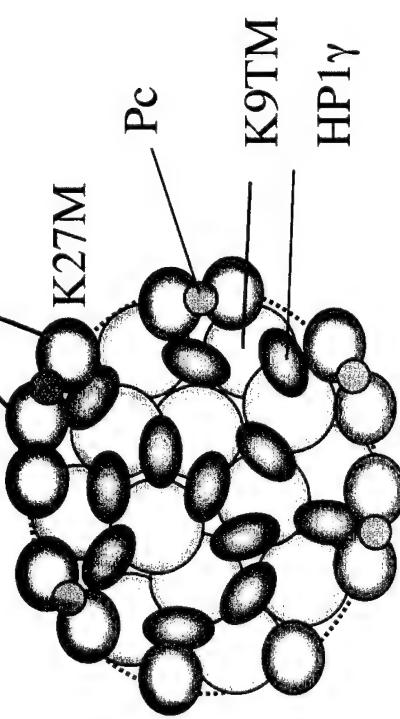
Senescent IMR90 cells were stained with anti-H3K27M and K9triM (A), or anti-K27M and K4M (euchromatin marker) antibodies (B). K27M form a “ring” structure, which includes K9triM core (A). K4M is excluded from K27M “ring” (B). Strong staining with K27M indicates inactive X chromosome (Xi).

Figure 2



B

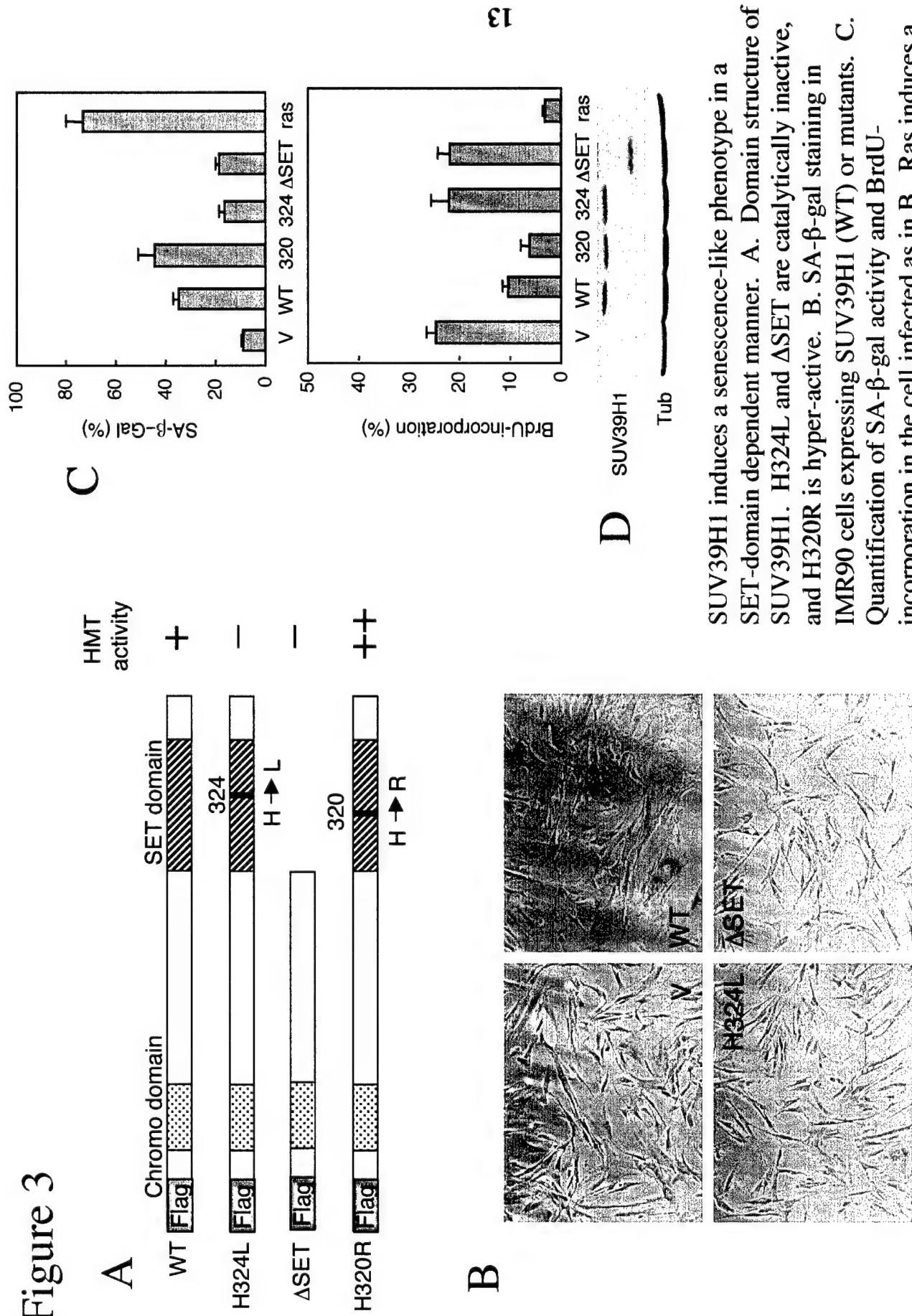
Transcription
(e.g. p16)



A. GFP-M33 (mouse *Pc1*) and H3 K27M colocalize in senescent IMR90 cells.

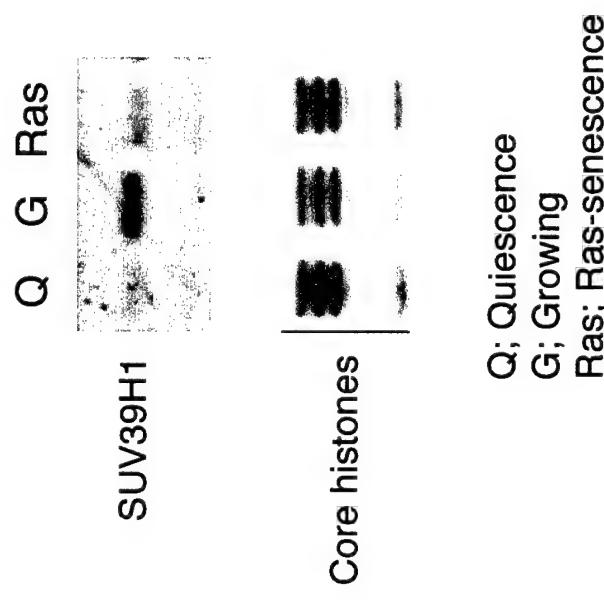
B. Molecular model of SAHF. The K27/Pc complex surrounds the K9triM/HP1 complex and transcription occurs outside of SAHFs.

Figure 3



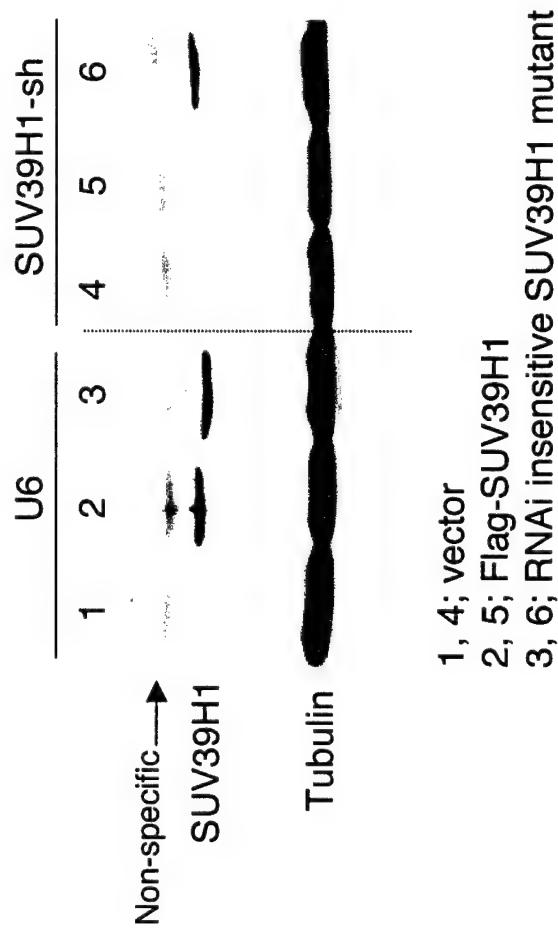
SUV39H1 induces a senescence-like phenotype in a SET-domain dependent manner. **A.** Domain structure of SUV39H1. H324L and Δ SET are catalytically inactive, and H320R is hyper-active. **B.** SA- β -gal staining in IMR90 cells expressing SUV39H1 (WT) or mutants. **C.** Quantification of SA- β -gal activity and BrdU incorporation in the cell infected as in B. Ras induces a higher percentage of senescent cells. **D.** Western blot analysis of SUV39H1 in the experiments in B and C.

Figure 4



SUV39H1 is enriched in the chromatin fraction of normal growing IMR90 cells. The chromatin fraction was isolated from quiescent (Q), growing (G), and Ras-senescence (Ras) cells, and blotted with anti-SUV39H1 antibody. Coomassie blue staining indicates equal amount of core histones.

Figure 5



IMR90 cells were co-infected with control vector, SUV39H1 or an RNAi insensitive mutant and either control (U6) or SUV39H1-short hairpin retroviruses. Western blotting reveals that the level of exogenous SUV39H1 is significantly decreased when the SUV39H1-sh is expressed.

Table 1. Production and characterization of anti-histone H3 K9M antibodies

Antibody	Species	Cross-reactivity with other H3 modification	Current status
H3 K9triM	rabbit	none	low titer
H3 K9diM	mouse	weakly with K9monoM	low titer. Needs further concentration
H3 K9diM	mouse	K9triM	under recloning
H3 K9triM	mouse	K27triM	affinity purified

Table 2. Candidates for the HMTs involved in senescence

Enzyme	targets
SUV39H1	H3 K9triM
SETDB1	H3 K9M
EZH2	H3 K9/27M
G9a	H3 K9/27M

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Narita M, Lowe SW. Executing Cell Senescence. *Cell Cycle*, 2004 Mar;3(3):244-246

Meeting abstract Era pf Hope 2002

SAHR; A NEW MARKER OF SENESCENCE

Masashi Narita, Edith Heard, Stephen Hearn, Masako Narita, Scott Lowe

There is ample evidence indicating that p53 plays a central role in tumor suppression in breast carcinogenesis. As a tumor suppressor, p53 is involved in both the apoptosis and senescence programs. Similar to apoptosis, various stimuli, including oncogenic ras and DNA damage, can induce a senescent phenotype, suggesting the existence of a common senescence machinery. The senescent phenotype continuously progress over the cell divisions, presumably without genetic alteration, which is strongly suggestive of epigenetic regulation in senescence program. While characteristic morphological changes in senescent cells are well known, the nuclear morphology of senescent cells is poorly characterized.

Human diploid fibroblasts, such as IMR90s, are well-established model system for cellular senescence. We have used this system along with retroviral gene transfer to characterize the nuclear morphology of senescent cells. IMR90s induced to senesce by Ras, as well as replicative senescent fibroblasts, show a characteristic DAPI staining pattern, which we have designated as SAHR, Senescence Associated Heterochromatic Regions. We have found a close correlation between SAHR formation and senescence markers such as, accumulation of p53/p16. Furthermore, the adenoviral oncoprotein E1A, which overrides senescence, also inhibits SAHR formation, and SAHRs are not found in quiescent cells. Taken together, these results indicate that SAHRs are a marker of senescence.

Immunofluorescence studies reveal that SAHRs colocalize with markers of heterochromatin, such as HP1 and Lys9 methylated histone H3. In contrast, lys9/14 acetylation and lys4 methylation of H3, which are consistent with euchromatin, are excluded from SAHRs. Electronmicrography confirms similar heterochromatic structures in senescent cells, where DNA is condensed and RNA is absent, suggesting that SAHRs are a novel type of heterochromatic structure. Given the fact that the levels of acetylated H3 are downregulated in senescent cells, our data suggest a global rearrangement of chromatin structure during senescence. If this is true, it could define a specific gene expression pattern particular to senescence and provide insights into an epigenetic regulation as a common machinery of senescence-related tumor suppression.

Meeting abstract

11th International p53 Workshop, Barcelona, Spain, May 15-19, 2002

SAHR, a new marker of senescence, which exhibit heterochromatic features

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Cold Spring Harbor Laboratory

Human diploid fibroblasts (HDFs), such as IMR90s, enter an irreversible growth arrest after a limited number of divisions in culture, known as replicative senescence. Oncogenic ras induces premature senescence, which is phenotypically indistinguishable from replicative senescence. Cellular senescence is accompanied by the accumulation of the tumor suppressors p53 and p16. Both the p53 and p16/Rb pathways must be circumvented for HDFs to escape from senescence.

While characteristic morphological changes in senescent human fibroblasts are well known, the nuclear morphology of senescent cells is poorly characterized. Ras-induced senescent IMR90s, as well as replicative senescent fibroblasts, show a characteristic DAPI staining pattern, which we have designated as SAHR, Senescence Associated Heterochromatic Regions. We have found a close correlation between SAHR formation and senescence markers such as, accumulation of p53/p16 and senescence-associated β -galactosidase activity. Furthermore, the adenoviral oncoprotein E1A, which overrides senescence, also inhibits SAHR formation, and SAHRs are not found in quiescent cells. Taken together, these results indicate that SAHRs are a marker of senescence.

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Meeting abstract

Cancer Genetics and Tumor Suppressor Genes, CSHL, August 14-18, 2002

Poster

HIGHER ORDER CHROMATIN STRUCTURE AND CELLULAR SENESCENCE

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Human diploid fibroblasts (HDFs), such as IMR90s, enter an irreversible growth arrest after a limited number of divisions in culture, known as replicative senescence. Oncogenic ras induces premature senescence, which is phenotypically indistinguishable from replicative senescence. Cellular senescence is accompanied by the accumulation of the tumor suppressors p53 and p16. Both the p53 and p16/Rb pathways must be circumvented for HDFs to escape from senescence.

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Meeting abstract

Molecular Genetics of Aging meeting, CSHL, October 2-6, 2002
oral

CHROMATIN REORGANIZATION CONTRIBUTES TO SENESCENCE

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The replicative exhaustion of Human diploid fibroblasts (HDFs) in culture is associated with an “irreversible” cell-cycle arrest known as replicative senescence. However, young cells can enter a state of cell-cycle arrest that is phenotypically identical to cellular senescence in response to expression of certain mitogenic oncogenes, oxidative stress, and DNA damage. Both replicative and ‘premature’ senescence involve activation of the p53 and p16/Rb tumor suppressor pathways. Here we describe a novel type of chromatin structure that has features of heterochromatin and appears to contribute to the senescence state. Specifically, we show that senescent HDFs harbor acquired regions of condensed chromatin that is enriched for HP1 proteins and histone H3 methylated on lysine 9. The appearance of these features requires the retinoblastoma gene product (Rb), since activation of Rb by enforced expression of p16 induces both senescence and chromatin alterations, and disruption of Rb function using viral oncoproteins prevents chromatin alterations in response to senescence-inducing stimuli. Interestingly, senescent cells display a defect in their ability to activate cell-cycle genes (relative to quiescence) in response to enforced expression of E2F, and disruption of Rb alleviates this repression. Moreover, enforced expression of SUV39H1, a histone H3 lysine 9 methyltransferase, induces premature senescence in HDFs in a manner that can be prevented by viral oncoproteins that target Rb. We propose that Rb-dependent changes in heterochromatic organization produce the specific gene expression patterns associated with senescence and, in particular, results in stable suppression of E2F target genes and a general non-responsiveness to mitogens.

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PUBLICATIONS

Original articles

Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW.

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Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence

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Summary

Cellular senescence is an extremely stable form of cell cycle arrest that limits the proliferation of damaged cells and may act as a natural barrier to cancer progression. In this study, we describe a distinct heterochromatic structure that accumulates in senescent human fibroblasts, which we designated senescence-associated heterochromatic foci (SAHF). SAHF formation coincides with the recruitment of heterochromatin proteins and the retinoblastoma (Rb) tumor suppressor to E2F-responsive promoters and is associated with the stable repression of E2F target genes. Notably, both SAHF formation and the silencing of E2F target genes depend on the integrity of the Rb pathway and do not occur in reversibly arrested cells. These results provide a molecular explanation for the stability of the senescent state, as well as new insights into the action of Rb as a tumor suppressor.

Introduction

Cellular senescence was originally described as the process of cell cycle arrest that accompanies the exhaustion of replicative potential in cultured human fibroblasts (Hayflick, 1965). Senescent cells remain metabolically active; display characteristic changes in cell morphology, physiology, and gene expression; and typically upregulate a senescence-associated β -galactosidase (SA- β -gal) activity (Campisi, 2001; Dimri et al., 1995; Shelton et al., 1999). Senescent cells are unable to express genes required for proliferation, even in a pro-mitogenic environment (Dimri et al., 1994, 1996). These features distinguish senescence from quiescence, a nonproliferative state that is readily reversed in response to mitogens. Although “replicative” senescence is triggered by telomere attrition, an identical endpoint (often

called “premature senescence” or “stasis”) can be acutely produced in response to activated oncogenes, DNA damage, oxidative stress, and suboptimal cell culture conditions. These observations imply that cellular senescence, like apoptosis, is a cellular response to stress that limits the proliferation of damaged cells (Campisi, 2001; Mathon and Lloyd, 2001).

Although cellular senescence is typically studied in cultured cells, the process may be important in aging and cancer (Campisi, 2001). Cellular senescence is often considered a cellular counterpart of organismal aging and, indeed, increases in SA- β -gal activity can be detected in cells from older individuals and patients with premature aging syndromes. Moreover, mutations that prevent DNA repair or promote chronic DNA damage can promote premature senescence *in vitro* and aging *in vivo*, and some genes that modulate senescence in cultured cells also affect lifespan in mice. Owing to its antiproliferative effects, senescence also appears to be a potent antitumor mechanism. Hence, mutations in certain tumor suppressor genes compromise senescence, thereby contributing to cell immortalization and cancer. Furthermore, cytotoxic agents used in cancer chemotherapy can induce cellular senescence, and defects in this process promote drug resistance *in vivo* (Chang et al., 1999; Schmitt et al., 2002; te Poel et al., 2002).

The Rb and p53 tumor suppressors are important senescence regulators. Rb and p53 are typically activated during senescence, and enforced expression of either protein induces senescence in some cell types (Ferbeyre et al., 2002; Lee et al., 2000). In human fibroblasts, DNA tumor virus oncoproteins that interfere with Rb and p53 function can bypass senescence. For example, SV40 large T antigen binds both Rb and p53 and overcomes replicative senescence, whereas large T mutants defective in binding either protein are less able to do so (Shay et al., 1991). Similarly, adenovirus E1A targets the Rb family and interferes with p53-mediated arrest and prevents senescence induced by oncogenic ras and DNA damaging agents (Lowe and Ruley, 1993; Serrano et al., 1997). In mouse embryo fibroblasts (MEFs), p53 loss is sufficient to overcome senescence, whereas inactivation of Rb alone has no obvious effect (Lowe and Sherr, 2003). Nevertheless, the Rb family contributes to senescence in this cell type, since cells lacking Rb along with the related p107 and p130 proteins fail to senesce in culture (Dannenberg et al., 2000; Sage et al., 2000).

In many instances, p53 and Rb are activated to promote senescence by products of the INK4a/ARF locus (Lowe and Sherr, 2003). This locus encodes two tumor suppressors, p16^{INK4a} and p14^{ARF} (p19^{ARF} in mice), expressed from partially overlapping nucleotide sequences read in alternative reading frames. p16^{INK4a} engages the Rb pathway by inhibiting cyclin D-dependent kinases that would otherwise phosphorylate and inactivate Rb. In contrast, p14^{ARF} increases the growth suppressive functions of p53 by interfering with its negative regulator, Mdm2. Both p16^{INK4a} and p14^{ARF} accumulate in senescent cells and can promote senescence when

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overexpressed (Lundberg et al., 2000). Moreover, mutations that affect *INK4a* or *ARF* can compromise senescence to varying degrees depending on species and cell type.

How Rb promotes senescence is not known. Rb family proteins are corepressors of the E2F transcription factors, and their combined activities are required for many aspects of cell cycle progression (Trimarchi and Lees, 2002). Rb-family members are thought to recruit histone deacetylases (HDACs) to E2F-dependant promoters, thereby deacetylating nearby histones and repressing gene expression. As cells approach S phase, cyclin D and E-dependent kinases phosphorylate Rb and free E2F, allowing it to act with histone acetyltransferases (HATs) to open chromatin structure and transactivate E2F-responsive genes important for G1 to S phase transition. The modification of histones by HATs and HDACs is dynamic and readily explains the reversibility of cell cycle arrest in quiescent cells. Recent studies suggest that p107 and p130, but not Rb, are associated with E2F-responsive genes during the cell cycle and quiescence (Rayman et al., 2002; Takahashi et al., 2000). Rb binds other proteins that influence gene expression, including the HP1 proteins and certain histone methyltransferases (Trimarchi and Lees, 2002). The relative contribution of these interactions to cell cycle regulation and Rb tumor suppressor functions has yet to be determined.

Although the molecular mechanisms underlying the irreversibility of cellular senescence remain poorly understood, these processes are extremely efficient. In fact, human fibroblasts almost never spontaneously escape replicative senescence and cannot be transformed unless the process is disabled (Campisi, 2001). Mutations that bypass cellular senescence can prevent the triggering event (e.g., telomerase activation) or act downstream of the damage signal to circumvent the senescence response (e.g., *INK4a* loss) (Brookes et al., 2002). Moreover, molecules that initiate senescence are often dispensable for its maintenance. For example, conditional expression of p53 and p16^{INK4a} can efficiently induce senescence in some settings, and the cells remain arrested after removal of the respective gene (Dai and Enders, 2000; Ferbeyre et al., 2002; Sugrue et al., 1997). In this study, we investigate the molecular basis for the stability of the senescent state and show that this occurs, in part, through an Rb-directed process that involves alterations in heterochromatin and the stable silencing of E2F target genes. We propose that these processes contribute to the tumor-suppressive properties of the senescence program.

Results

Changes in Nuclear Morphology and Chromatin Structure Accompany Senescence

In IMR90 human diploid fibroblasts undergoing senescence, we observed a characteristic nuclear morphology involving changes to the nucleolus and the organization of DNA. IMR90 cells induced to senesce by exogenous expression of oncogenic Ras, expression of activated MEK, treatment with the chemotherapeutic drug etoposide, enforced expression of p16^{INK4a}, or extensive passaging (i.e., replicative senescence), typi-

cally displayed one large nucleolus and punctate DNA foci as visualized by DAPI staining (Figure 1A; data not shown). In contrast, exponentially growing IMR90 cells, as well as cells made quiescent by serum withdrawal or confluence, usually displayed several small nucleoli and a more uniform DAPI staining pattern (Figure 1A; data not shown). IMR90 cells expressing E1A, which readily escape senescence, also did not develop pronounced nucleoli or DNA foci in response to Ras (Figure 1A). Finally, senescent WI38 cells, another normal fibroblast strain, acquired prominent DNA foci that appeared indistinguishable from senescent IMR90 cells (data not shown). Therefore, the changes to nuclear architecture are not unique to IMR90 cells, nor are they a necessary consequence of cell cycle arrest or oncogenic *ras* expression. Rather, these changes are specific for the senescent state.

In order to determine the kinetics of the appearance of senescence-associated DNA foci, we focused on the effects of oncogenic *ras*, as it acutely and reproducibly induces senescence in IMR90 cells over several days (Serrano et al., 1997). IMR90 cells were infected with retroviruses coexpressing oncogenic *ras* and a selectable marker (Figure 1B). After a brief drug selection to eliminate uninfected cells, cell populations were analyzed at various times for p16^{INK4a} and Rb expression, cellular proliferation (BrdU incorporation), the onset of senescence (SA- β -gal staining), and the appearance of DNA foci (DAPI staining). Cells expressing oncogenic *ras* accumulated p16^{INK4a} and hypophosphorylated Rb between 3 and 5 days postselection (Figure 1C), a time when these cells stopped incorporating BrdU and became positive for SA- β -gal (Figure 1D). Although control cells continued to proliferate and did not display senescence-related changes, the appearance of DNA foci in *ras*-expressing cells coincided precisely with cell cycle exit and the onset of senescence. Moreover, BrdU-positive nuclei containing DNA foci were never observed, implying that DNA synthesis and DNA focus formation were mutually exclusive processes.

Senescence-Associated DNA Foci Do Not Contain Active Sites of Transcription

To further characterize the nuclear changes that accompany senescence, normal and senescent IMR90 cells were examined by electron microscopy. In contrast to exponentially growing or quiescent controls (Figure 2A; data not shown), senescent nuclei showed prominent nucleoli, an irregular nuclear envelope, and electron-dense regions interspersed throughout the nucleoplasm (Figure 2B). To test whether these electron-dense regions correspond to the DNA foci observed by DAPI staining, we immunolabeled quiescent and senescent cells with an anti-DNA antibody and a secondary antibody conjugated to colloidal gold. In contrast to the diffuse pattern observed in quiescent cells, DNA labeling was concentrated in the electron-dense regions of senescent nuclei (Figure 2, compare C and D).

We also examined the relationship between DNA foci and sites of transcription. To assess global RNA localization, senescent cells were labeled with gold-conjugated RNase, which binds to and localizes RNA following electron microscopy. RNA was mostly excluded from the

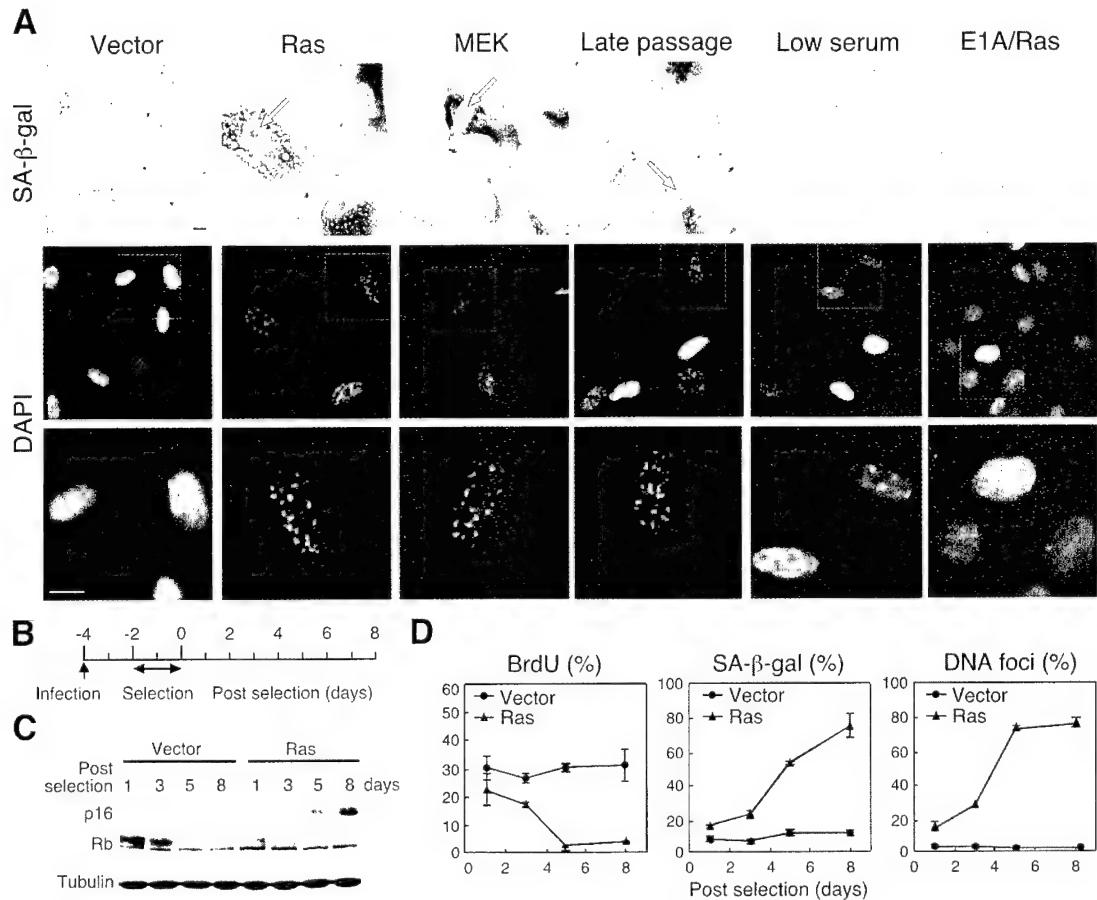


Figure 1. DNA Foci Accumulate in Senescent Cells

(A) IMR90 cells containing empty vector, H-rasV12 (Ras), MEK1 Q56P (MEK), or E1A12S/H-rasV12 (E1A/Ras) were stained for SA- β -gal activity, a classical marker of senescence, followed by DAPI staining, 6 days postselection. Late passage and quiescent cells, induced by culture in 0.1% serum for 48 hr (Low serum), are also shown for comparison. Enlarged images of DAPI staining are shown in the lower panels. Arrows indicate prominent nucleoli. Scale bars are equal to 10 μ m.

(B) Experimental design and time frame. For cells that were serially selected, day zero is set after the first selection.

(C) Protein expression of p16^{INK4a} and Rb was assessed by Western blotting using lysates from cells containing empty vector or H-rasV12 (Ras) over a period of days. Tubulin serves as a loading control.

(D) IMR90 cells containing empty vector or H-rasV12 (Ras) were scored for percentage of BrdU incorporation (left image), SA- β -gal activity (middle image) and the presence of DNA foci (right image), at the indicated days post selection.

interior of the large DNA foci and was instead interspersed throughout the nucleoplasm and concentrated in small foci (Figure 2D). The latter structures may correspond to perichromatin fibrils or RNA-containing nuclear bodies, known to be active transcription sites (Spector, 2001). To localize specific gene transcription, we performed RNA fluorescence *in situ* hybridization (RNA FISH) using bacterial artificial chromosome (BAC) clones harboring the *cyclin A* and *INK4a* genes as probes. *Cyclin A* is expressed in dividing cells and down-regulated during senescence. As expected, two *cyclin A* signals were observed in exponentially growing cells (Figure 2E, vector). In cells undergoing senescence, these two signals were often observed at the periphery of DNA foci and subsequently extinguished (Figure 2E, compare Ras PS1 to Ras PS7). In contrast, *INK4a* is silent in dividing cells but upregulated during senescence. Concordantly, no *INK4a* RNA FISH signal was observed in growing cells, while two signals appeared in most senescent cells (Figure 2E, compare vector to

Ras PS7). In all cases, the positive signals were either at the periphery or outside of the DNA foci. Hence, senescence-associated DNA foci are condensed regions of DNA that correlate with transcriptionally inactive sites.

Senescence-Associated DNA Foci Have Features of Heterochromatin

The senescence-associated DNA foci are reminiscent of heterochromatin, which encompasses transcriptionally inactive regions of the genome that are packaged into highly dense chromatin fibers during interphase. Heterochromatin is important for various nuclear functions, including chromosome segregation, nuclear organization, and gene silencing (Henikoff, 2000; Jenuwein, 2001). At the molecular level, heterochromatic regions often lack histone H3 that is acetylated on lysine 9 (K9Ac-H3) and methylated on lysine 4 (K4M-H3). In contrast, these regions are usually enriched for histone H3 methylated on lysine 9 (K9M-H3). Notably, methylated lysine 9 provides a docking site for HP1 proteins (Ban-

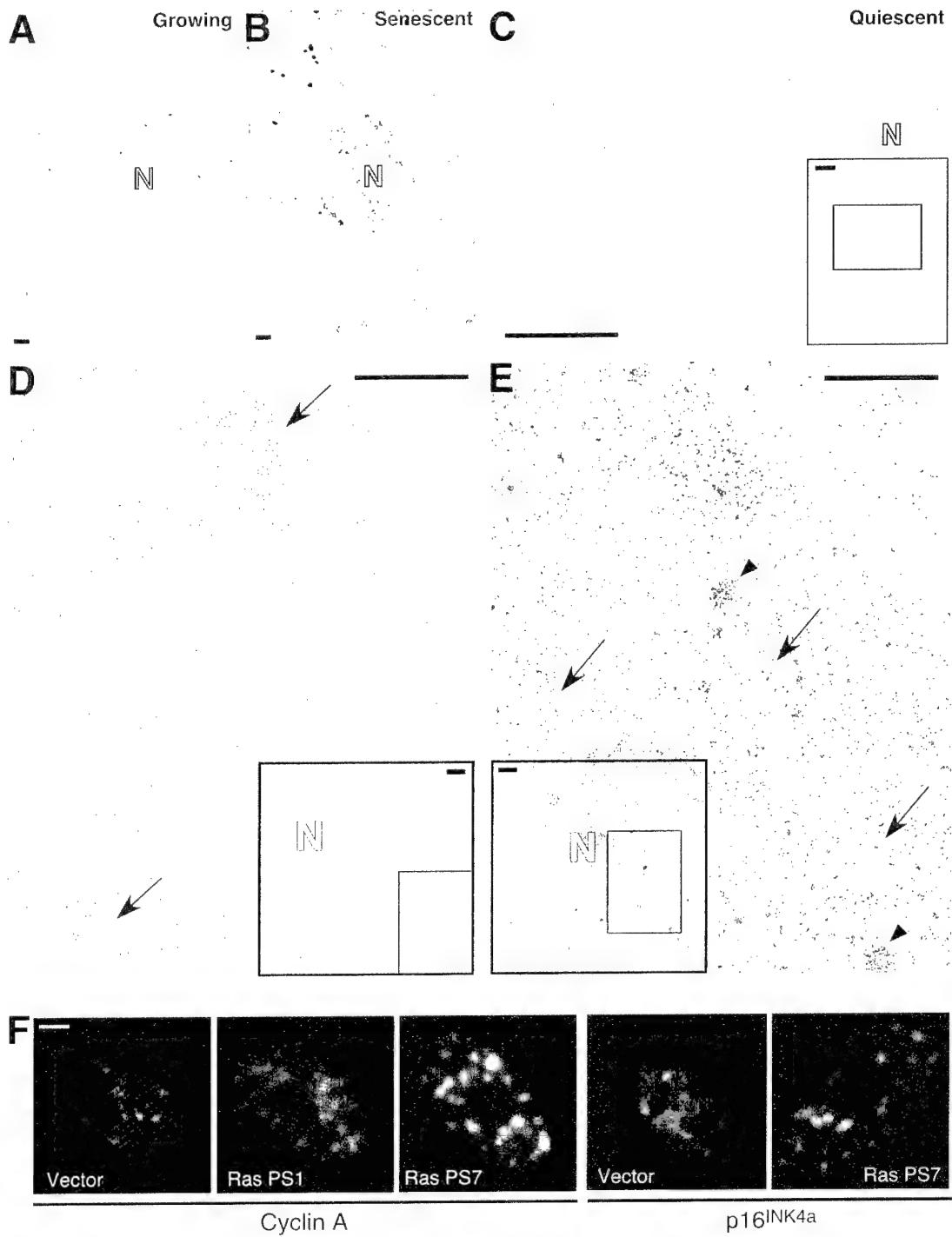


Figure 2. Senescence-Associated DNA Foci Are Not Sites of Active Transcription

(A and B) Electron microscopy images of vector control (Growing) (A) and Ras-senescent IMR90 cells (senescent) (B) "N" is nucleolus. Scale bars are equal to 1 μ m.

(C) High magnification of a nucleus from low serum quiescent cells showing DNA detected using a monoclonal anti-DNA antibody and gold-coupled secondary antibody. Scale bar is equal to 1 μ m.

(D) Higher magnification of a senescent nucleus (from rectangle in inset) showing DNA detected using a monoclonal anti-DNA antibody and gold-coupled secondary antibody. Arrows show the corresponding higher electron density regions of (B). Scale bar is equal to 1 μ m.

(E) Senescent nucleus showing RNA labeled with gold-coupled RNase T1 at same magnification as (D). Arrows show electron-dense regions corresponding to DNA foci in (B). RNA-rich small foci are likely to represent perichromatin fibrils or RNA-containing nuclear bodies (arrowheads). Scale bar is equal to 1 μ m.

(F) RNA FISH for *Cyclin A* and *INK4a* (p16^{INK4a}) was performed on vector control and Ras-senescent IMR90 cells (Ras) at the indicated post selection (PS) days. Intense signals of *INK4a* in Ras-senescent cells were localized at the periphery or outside of the DNA foci. DNA was counterstained by DAPI, which was pseudocolored green. Scale bar is equal to 5 μ m.

nister et al., 2001; Lachner et al., 2001), a family of adaptor molecules that are required for heterochromatin assembly and are involved in epigenetic gene regulation.

To determine whether the senescence-associated DNA foci are related to heterochromatin, we conducted confocal fluorescence microscopy on growing, quiescent, and senescent cells using modification-specific antibodies against histone H3 or the HP1 proteins α , β , and γ . Growing and quiescent cells expressed histones with all modifications examined, and these appeared distributed throughout the nucleoplasm (Figure 3A, vector and low serum). In marked contrast, senescent cells showed a more distinctive localization of modified histones. Consistent with their preference for euchromatic regions, K9Ac-H3 and K4M-H3 were largely excluded from DNA foci. Conversely, K9M-H3 was concentrated in the DNA foci of senescent cells (Figure 3A, Ras and Late passage). Similarly, all three HP1 proteins were dispersed throughout the nucleoplasm in normal and quiescent cells but were concentrated to varying degrees in the DNA foci of senescent cells (Figure 3B). This distinctive pattern was not due to variable antibody accessibility, since IMR90 cells expressing a green fluorescence protein (GFP)-HP1 β fusion protein also showed a GFP pattern that was dispersed in normal cells but concentrated in DNA foci of senescent cells (Supplemental Figure S1A available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>). Consequently, senescence-associated DNA foci contain heterochromatin and were defined as "senescence-associated heterochromatic foci" (SAHF).

Constitutive heterochromatin consists of large regions of heterochromatin found near the centromeres and telomeres of mammalian chromosomes during interphase. In principle, the SAHFs might result from a redistribution of preexisting heterochromatin (e.g., owing to changes in nuclear structure) rather than from new heterochromatin that accumulates in senescent cells. However, several observations suggest that this is unlikely. First, no substantial colocalization was observed between SAHF formation and either centromeres or telomeres, as determined by immunofluorescence using autoserum that recognizes centromeres or an antibody directed against telomere associated protein TRF2, respectively (Supplemental Figure S1B available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>). Second, senescent cells (induced by oncogenic *ras*) displayed a substantial increase in the total amount of chromatin-bound HP1 β and HP1 γ relative to normal proliferating and quiescent cells (Supplemental Figure S1C available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>). Importantly, E1A abolished the accumulation of chromatin-bound HP1 in response to oncogenic *ras* expression, indicating that these changes were linked to the senescent state. Finally, consistent with the known resistance of heterochromatin to limited nuclelease digestion (Leuba, et al., 1994), DNA from senescent cells was more resistant to micrococcal nuclease compared to normal cells and E1A-expressing cells that had bypassed *ras*-induced senescence (Figure 3C). Together, these results imply that formation of a distinct heterochromatic structure accompanies the senescence process.

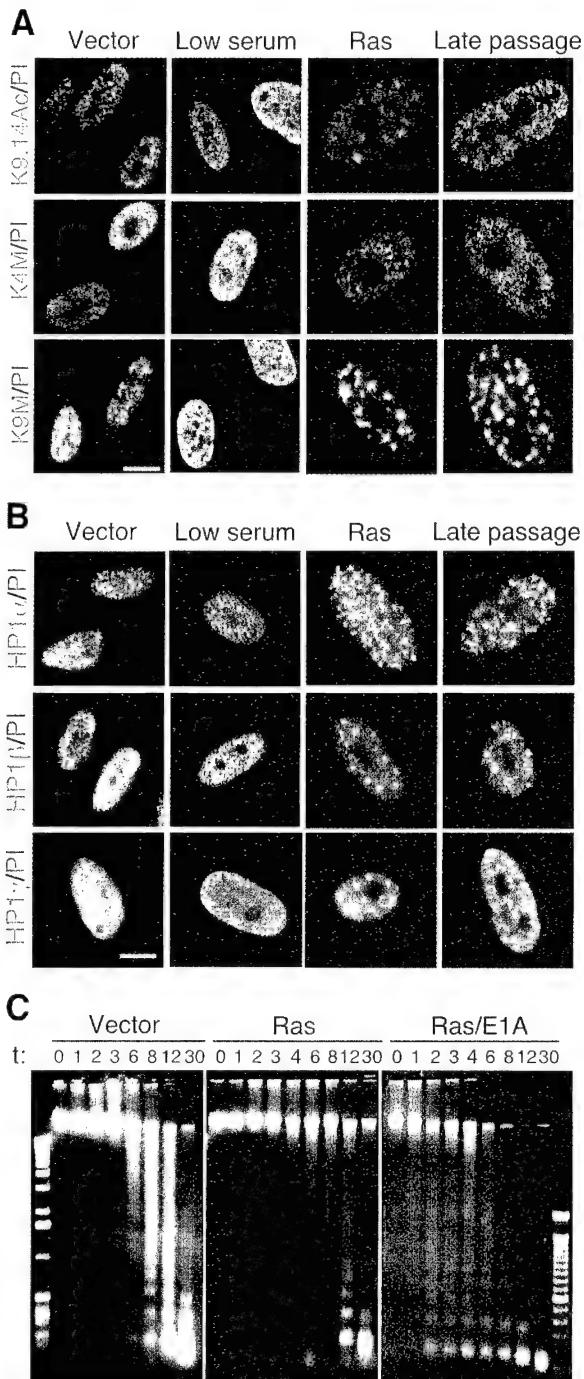


Figure 3. Senescent Cells Accumulate Features of Heterochromatin
(A) Confocal images of indirect immunofluorescence of acetylated histone H3 on Lysine 9/14 (K9/14Ac), methylation on lysine 4 (K4M), and methylation on lysine 9 (K9M) (green) in normal growing (vector), low serum quiescent (low serum), H-rasV12 (Ras) senescent, or replicative senescent IMR90 cells (late passage). The DNA was counterstained by propidium iodide (PI) (red). Merged images are shown.
(B) Localization of endogenous HP1 proteins (green) was determined by indirect immunofluorescence using the indicated antibodies in the cells described in (A). DNA was stained with propidium iodide (PI) (red). Merged images are shown.
(C) Micrococcal nuclease digestion of detergent-permeabilized cells from growing (Vector), senescent (Ras), and Ras/E1A transformed cells. DNA was isolated from cells after digestion for the indicated time (min) and subjected to agarose gel electrophoresis.

Some E2F Target Promoters Acquire Heterochromatic Features during Senescence

The changes in euchromatic and heterochromatic organization that accompany SAHF formation might produce senescence-specific changes in gene expression. Since heterochromatin has been linked to gene silencing, we reasoned that SAHFs might contribute to the stable cell cycle arrest that is a hallmark of senescence. If true, then components of euchromatin or heterochromatin would be associated with genes that are induced or repressed during senescence, respectively. To test this, we examined the association of K9/14Ac-H3, K9M-H3, and HP1 γ to the promoters of several genes in senescent cells *in vivo* using chromatin immunoprecipitation. We focused on E2F-target promoters because these genes are essential for cell cycle progression and are negatively regulated by the Rb family which, in turn, influences senescence. These genes are also constitutively induced by E1A, which inactivates Rb and prevents SAHF formation and senescence. Here, we analyzed senescent cells produced by oncogenic *ras*, because these cells undergo a relatively synchronous arrest.

We first examined the binding of K9/14Ac-H3, a marker of euchromatin, for its presence on genes associated with senescence. *Stromelysin-1* and *INK4a* are upregulated during senescence, whereas *cyclin A* and *PCNA* are E2F-target genes that are repressed. Compared to normal cells, the amount of K9/14Ac-H3 bound to the *stromelysin-1* promoter increased in senescent cells, although no change was observed on the *INK4a* promoter (Figure 4A, compare lanes 2 and 3). In contrast, the amount of K9/14Ac-H3 that associated with *cyclin A* and *PCNA* declined during senescence. Interestingly, E1A abolished the increase in K9/14Ac-H3 binding to the *stromelysin-1* promoter, but produced an increase in K9/14Ac-H3 association with the *cyclin A* and *INK4a* promoters (Figure 4A, compare lanes 3 and 4). These latter results are consistent with the known E2F responsiveness of *cyclin A*, and the observation that *INK4a* levels often increase following Rb inactivation (Stott et al., 1998). The amount of K9/14Ac-H3 bound to the *stromelysin-1* promoter did not increase in quiescent cells (produced by serum depletion or confluence), indicating that this effect was specific for senescence (Figure 4B, compare lane 2 to lanes 3 and 4). However, the amount of K9/14Ac-H3 bound to the *cyclin A* and *PCNA* promoters also declined in quiescent cells, indicating that hypoacetylation of histones on E2F target promoters is not unique to senescence.

We next examined the occupancy of E2F target promoters by K9M-H3 and HP1 γ : two proteins that are enriched in the SAHFs and are known to be involved in heterochromatin formation (Bannister et al., 2001; Lachner et al., 2001). In marked contrast to acetylated histone H3, the amount K9M-H3 associated with the *cyclin A* and *PCNA* promoters increased in senescent cells relative to quiescent cells (Figure 4C, compare lanes 1 and 2). Although the amount of K9M-H3 detected from experiment to experiment was variable, similar results were produced with two anti-K9M-H3 antibodies. Similarly, HP1 γ , which binds K9M-H3 in the context of heterochromatin, also associated with the *PCNA* and *cyclin A* promoters in senescent but not quiescent cells (Figure 4C, compare lanes 3 and 4). Importantly, E1A,

which prevents senescence and SAHF formation, also prevented *ras*-induced HP1 γ and K9M-H3 accumulation on the two E2F target promoters (Figure 4D, compare lanes 1–2 and 7–8). Therefore, the accumulation of K9M-H3 and HP1 γ on the promoters examined is not detected when cells exit the cell cycle into a reversible quiescent state, but accompanies the more stable senescence-like arrest.

Rb Associates with E2F-Responsive Promoters in Senescent but Not Quiescent Cells

Rb is an important regulator of E2F-responsive genes and contributes to cellular senescence (Campisi, 2001). Although Rb is often linked to normal cell cycle progression, recent reports indicate that p107 and p130 are the predominant Rb family members bound to E2F responsive promoters in quiescent and G1 cells (Rayman et al., 2002; Takahashi et al., 2000). Interestingly, in addition to the E2Fs, Rb can also associate with HP1 and histone methyltransferases such as SUV39H1, raising the possibility that Rb helps direct the process of histone methylation and HP1 recruitment to E2F responsive promoters during senescence. Consistent with this possibility, Rb showed a limited colocalization with SAHFs in the nuclei of senescent cells, which was greater than that observed for p107 and p130 (Supplemental Figure S2 available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>).

To determine whether Rb might occupy E2F target gene promoters during senescence, we examined the association of all three pocket proteins with the *cyclin A* and *PCNA* promoters in quiescent (confluent) and senescent (*ras*) cells using chromatin immunoprecipitation. As expected, p107 and p130 were readily detected on the *cyclin A* and *PCNA* promoters in quiescent cells (Figure 4E, lanes 1 and 3). While the association between p107 and these promoters typically declined in senescent cells, p130 was retained to varying degrees (Figure 4E, lanes 2 and 4; data not shown). In contrast, Rb was difficult to detect on the *cyclin A* and *PCNA* promoters in quiescent cells, but was detected on these promoters in senescent cells (Figure 4E, compare lanes 5 and 6). Although the Rb signal in senescent cells was often weak (perhaps owing to antibody inaccessibility to the Rb localized to compacted chromatin), we observed similar results in multiple experiments (data not shown). These results indicate that Rb can physically associate with some E2F target promoters in senescent cells and raise the possibility that Rb plays a special role in this process.

E2F-Responsive Genes Are Stably Repressed in Senescent Cells

In principle, the altered chromatin state accompanying SAHF formation and the binding of K9M-H3, HP1, and Rb to E2F responsive promoters could stably silence the expression of E2F responsive genes and produce a permanent insensitivity to mitogenic signals. Indeed, in contrast to quiescent cells, mitogenic growth factors are unable to activate E2F target genes in senescent cells (Good et al., 1996). Furthermore, consistent with their incorporation into a more condensed chromatin state, we have observed that some E2F target genes are more resistant to MNase digestion in senescent compared to quiescent cells (see Supplemental Figure

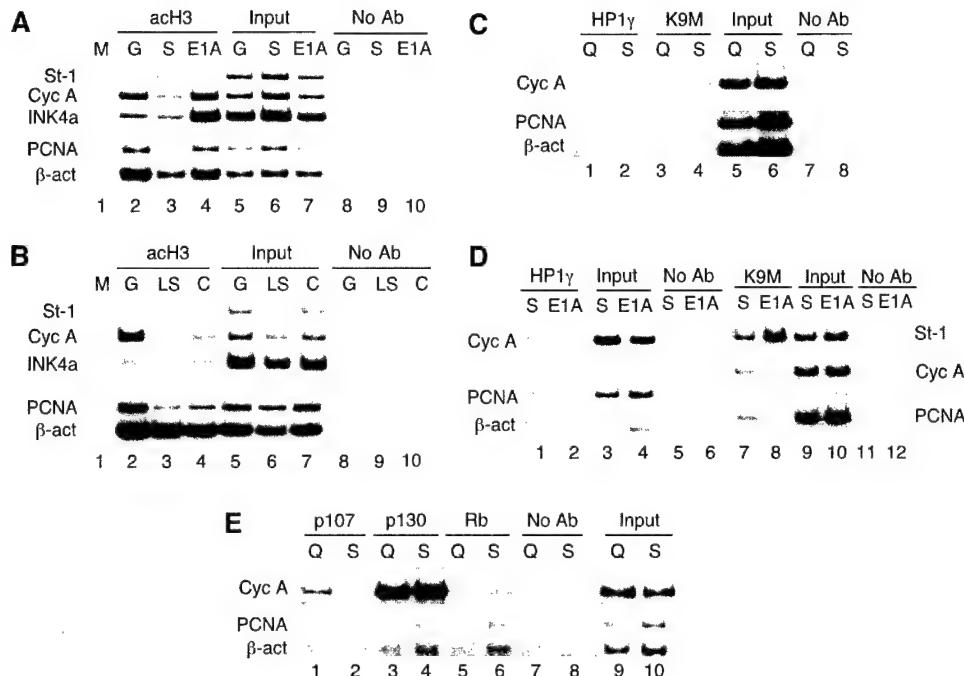


Figure 4. Chromatin Immunoprecipitation Analysis of E2F Target Promoters in Quiescent and Senescent Cells

(A and B) Chromatin immunoprecipitation assay (ChIP) using either an antibody against acetylated histone H3 (acH3), no antibody (No Ab) or nuclear extract (input). DNA fragments were amplified by PCR from the promoter regions of *stromelysin-1* (St-1), *cyclin A* (Cyc A), *INK4a*, *PCNA*, and β -actin (β -act). Buffer without nuclear extract served as Mock (M) control. Normal growing IMR90 cells (G), Ras-senescent cells (S), and E1A/Ras transformed cells (E1A) were used in (A); normal growing cells (G), low serum (LS), and confluent quiescent cells (C) were used in (B). DNA fragments were amplified by PCR from the promoter regions of *cyclin A* (Cyc A), *PCNA*, and β -actin (β -act).

(C and D) ChIP assays were performed using HP1 γ and K9M-H3 antibodies on extracts from quiescent (Q), Ras-senescent (S), and E1A/Ras-expressing (E1A) IMR90 cells.

(E) ChIP assays were performed as in (C) using p107, p130, or Rb antibodies on extracts from quiescent (Q) and Ras-senescent (S) cells.

S3 available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>). To examine this insensitivity directly, we introduced E2F-1 into quiescent (serum-starved) or senescent (ras) cells using adenovirus-mediated gene transfer and examined the expression of several E2F target genes using quantitative real-time RT-PCR 48 hr after infection. This approach allowed us to circumvent the mitogenic signaling pathways that are otherwise required for E2F activation.

Consistent with previous reports (Leone et al., 1998), expression of E2F-1 in quiescent cells produced a substantial increase in *MCM3* and other E2F-targets, *cyclin A*, *PCNA*, and *DHFR* expression (Figure 5A). This effect was a result of E2F-1 and not adenovirus infection, since a control adenovirus did not induce these E2F targets. In contrast, E2F-1 was unable to effectively induce E2F target genes in senescent cells (Figure 5A), despite similar levels of E2F-1 expression (Figure 5B; data not shown). Although some E2F target genes could be induced in senescent cells at high concentrations of E2F-1 (data not shown), their relative insensitivity to E2F-1 was observed over a substantial concentration range (Figure 5B). Therefore, E2F target genes are stably repressed in senescent cells.

The p16^{INK4a}/Rb Pathway Is Required for SAHF Formation and E2F-Target Silencing

The potential role of Rb in directing SAHF formation and the silencing of E2F target genes may explain, in part,

its role in controlling senescence. The E1A oncoprotein, which can contribute to cellular immortalization, counters Rb function and prevents SAHF formation (see Figure 1A). Interestingly, Rb interacts with HP1 proteins via its "LXCXE" motif, and this interaction can be disrupted by the E1A oncoprotein (data not shown). To further characterize the impact of the Rb family on SAHF formation and E2F target gene silencing, we examined the ability of E1A to affect cellular proliferation (BrdU incorporation), SA- β -gal activity, SAHF formation, and the expression of E2F target genes in response to oncogenic ras. In some experiments, we replaced oncogenic ras with p16^{INK4a}, which engages the Rb pathway and is sufficient to induce senescence in normal diploid fibroblasts (Brookes et al., 2002).

As expected, full-length E1A abrogated ras-induced senescence (Figure 6). Hence, E1A-expressing cells continued to proliferate, did not accumulate SA- β -gal activity or SAHFs, and expressed high levels of E2F-responsive genes (Figure 6C, see also Figure 1A). Interestingly, E1A Δ N, which preserves the LXCXE motif and targets the Rb family but not the p300 and p400 proteins, was not as effective as full-length E1A at preventing cell cycle arrest or SA- β -gal accumulation (Figures 6A and 6B), but retained its ability to prevent SAHF formation and repression of E2F targets (Figures 6B and 6C). Enforced expression of p16^{INK4a} also induced SA- β -gal activity, SAHF formation, and repressed E2F target genes and, in this setting, E1A Δ N was able to completely reverse the phenotype (Figures 6A and 6B). Finally, cells

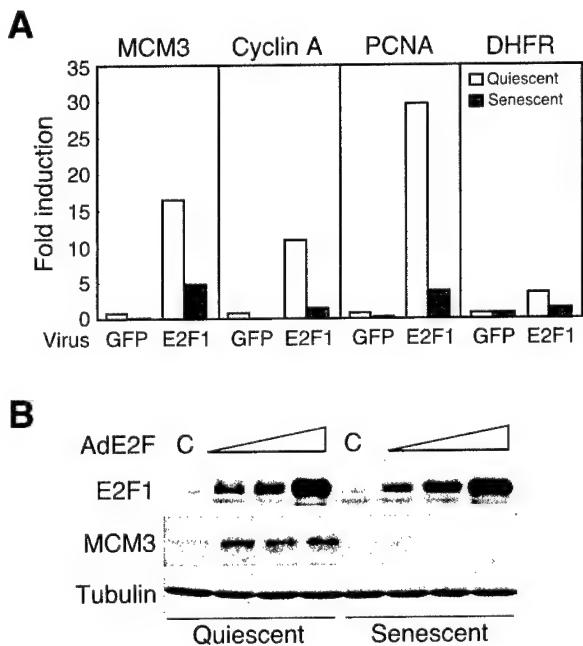


Figure 5. E2F Target Genes in Senescent Cells Are Resistant to Activation by E2F

(A) Quiescent (low serum) and Ras-senescent IMR90 cells were infected with adenoviruses expressing GFP and E2F-1. MCM3, Cyclin A, PCNA, and DHFR levels were determined by real-time RT-PCR.

(B) Quiescent (by confluence) and Ras-senescent IMR90 cells were infected with adenoviruses expressing GFP and E2F-1. MCM3 level was determined by Western blotting (triangle indicates increasing multiplicities of infection).

expressing a dominant-negative p53 underwent cell cycle arrest, accumulated SAHF, and downregulated E2F targets (Figure 6), indicating that these aspects of the senescent program can occur independently of p53. Therefore, activation of one or more Rb-family proteins is necessary, and perhaps sufficient, for SAHF formation and the stable repression of E2F target genes.

The fact that Rb preferentially associates with specific E2F target promoters during senescence suggests that it may play a particularly important role in SAHF formation and the silencing of E2F target promoters. Although the E1A data are consistent with this view, E1A also binds p107 and p130, and these proteins can also associate with E2F target promoters in senescent cells. To directly target Rb, we exploited stable RNA interference (RNAi) technology to specifically knock down p16^{INK4a} and Rb expression. A series of short hairpin RNAs (shRNAs) targeting different sequences in the p16 and Rb mRNAs were generated that had ≥ 6 nucleotide differences from any other known human gene, and several were identified that efficiently suppressed p16^{INK4a} or Rb expression (data not shown). Next, IMR90 cells were infected with retroviruses expressing an shRNA directed against p16^{INK4a} (sh-p16) or Rb (sh-Rb), in combination with a *ras*-expressing retrovirus to induce senescence. Both p16^{INK4a} and Rb were stably repressed by their respective shRNA relative to the control vector (Figure 7A, compare lanes 4 to 5 for p16; compare lanes 4–6 for Rb). Interestingly, like E1A Δ N, neither sh-p16 nor sh-

Rb prevented eventual cell cycle exit, but both reduced SAHF formation (Figure 7B) and prevented the silencing of E2F targets (Figure 7A, compare lane 4–5 and 6 for MCM3, cyclin A, and PCNA).

These effects were more dramatic when examined at the single-cell level (Figure 7C). As expected, most cells coexpressing *ras* with a control shRNA vector contained SAHFs, as assessed by DNA foci and punctate HP1 γ staining. These cells also expressed low levels of MCM3, which is an E2F-regulated gene, critical for the initiation of DNA replication. In contrast, cells coexpressing E1A Δ N did not produce SAHFs and expressed extremely high MCM3 levels. Cells coexpressing sh-p16 or sh-Rb were more heterogeneous; whereas many cells did not form SAHFs and expressed high MCM3 levels, a subset developed SAHFs and silenced MCM3. Hence, a close correlation exists between SAHF formation and the repression of the E2F target genes examined, which is consistent with a stable silencing of these genes. These data demonstrate that Rb controls heterochromatin structure and gene silencing in senescent cells and provide an explanation for the stability of the senescent state.

Discussion

Cellular senescence is considered a permanent form of cell cycle arrest that is characterized by distinct changes in gene expression and an extreme insensitivity to mitogenic stimuli. We show that senescent IMR90 human fibroblasts accumulate a distinct chromatin structure enriched with heterochromatin proteins (designated SAHF) that excludes active transcription and is characterized by the accumulation of K9M-H3 and HP1 proteins. We further show that heterochromatin-associated proteins and the Rb tumor suppressor can accumulate on the E2F-responsive promoters in senescent but not quiescent cells, and that these changes are associated with more stable repression of E2F responsive genes. Notably, Rb is required for SAHF formation and E2F target gene silencing. We suggest that Rb-directed changes in heterochromatin organization contribute to senescence-associated changes in gene expression and the permanence of the senescent state.

Senescent Cells Accumulate a Distinctive Type of Heterochromatin

The organization of DNA into heterochromatin contributes to nuclear organization, chromosome structure, and gene silencing (Dillon and Festenstein, 2002; Lachner and Jenuwein, 2002). Constitutive heterochromatin primarily encompasses the pericentric regions of chromosomes and is important for chromosome segregation and the silencing of repetitive elements. Facultative heterochromatin is developmentally controlled and contributes to gene regulation during differentiation and influences dosage compensation. Here, we identify a distinctive type of facultative heterochromatin—designated SAHF—that accumulates in senescent cells. SAHFs are observed in interphase nuclei and contain the heterochromatin-associated proteins K9M-H3 and HP1, exclude histone modifications found in euchromatin (e.g., K9Ac-H3 and K4M-H3), and are not sites of

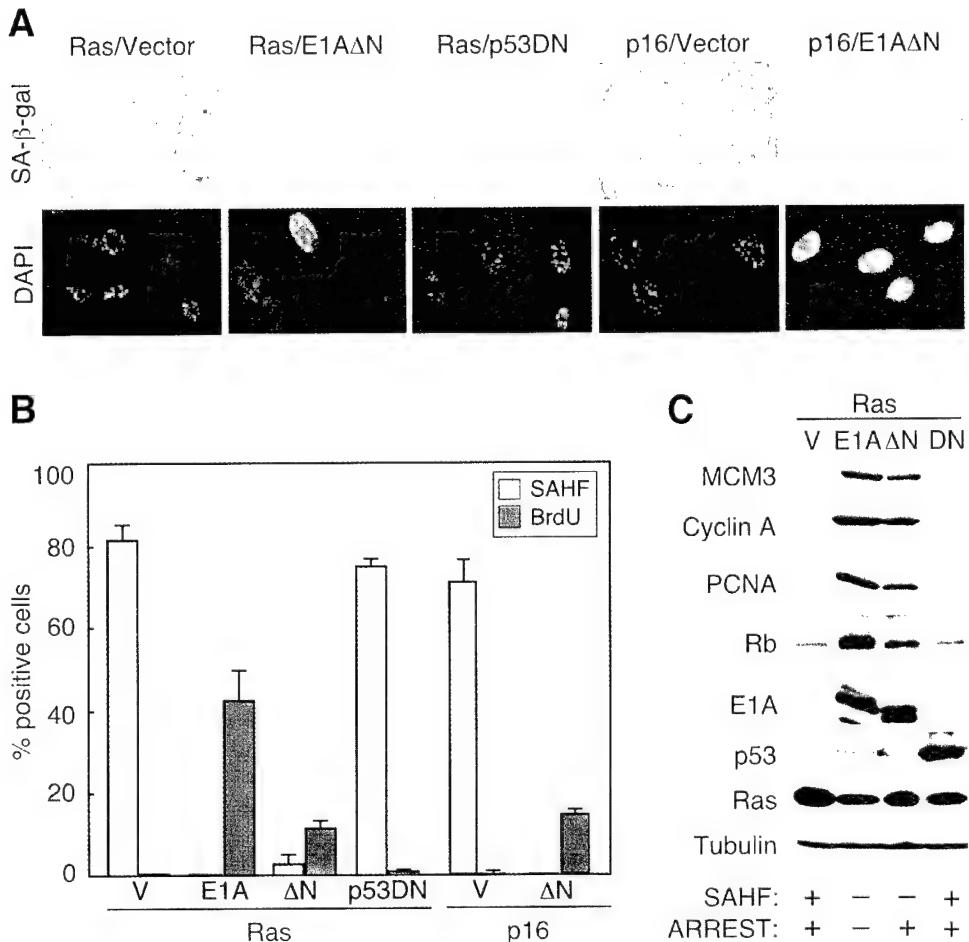


Figure 6. Inactivation of the Rb Family Proteins by E1A Prevents SAHF Formation and Gene Silencing

(A) IMR90 cells expressing H-rasV12 (Ras) in combination with either vector (vector), E1A Δ N (lacking 2–36 amino acids), or p53^{175H} (p53DN), and IMR90 cells expressing p16^{INK4a} in combination with either vector or E1A Δ N were stained for SA- β -gal activity at day 6 (see Figure 1B). DAPI staining is shown below.

(B) Same cells as (A), as well as IMR90 cells expressing both E1A and H-rasV12 were assessed for SAHF formation and BrdU incorporation at the same time point as (A).

(C) The expression of the indicated protein was determined by immunoblotting using extracts from IMR90 cells expressing H-rasV12 (Ras) in combination with either empty vector (V), E1A, or E1A Δ N (Δ N), a p53 dominant-negative (DN) at the same time point as in (A) and (B).

active transcription. SAHFs are distinct from pericentric heterochromatin, and their appearance is accompanied by an increase in HP1 incorporation into senescent chromatin and an enhanced resistance of senescent DNA to nuclease digestion.

SAHF formation requires an intact Rb pathway, since expression of E1A, or inactivation of either p16^{INK4a} or Rb, can prevent their appearance. During the initial phases of senescence, Rb might control the nucleation of heterochromatin at specific sites throughout the genome, which then spreads by the action of histone methyltransferases and recruitment of HP1 proteins. HP1 proteins have the capacity to dimerize and may interact to form higher order chromatin structures once a critical mass has been reached (Brasher et al., 2000; Nielsen et al., 2001a). A similar pattern of nucleation and spreading occurs during silencing of the mating type locus in *S. pombe*, position effect variegation in *Drosophila*, and X inactivation in mammalian cells, although HP1 proteins do not accumulate on the inactive X (Heard et al., 2001;

Peters et al., 2002). Importantly, SAHF formation correlates precisely with cell cycle exit and the silencing of E2F target genes.

Our results suggest that SAHFs causally contribute to cellular senescence, at least in part, by controlling the stability of the arrest. In this study we characterized the formation of SAHFs in IMR90 human diploid fibroblasts, a cell type where cellular senescence has been studied extensively. Interestingly, SAHF formation also occurred in senescent WI38 human fibroblasts, but was less pronounced in senescent BJ human fibroblasts and MEFs (data not shown). These differences are noteworthy in light of recent reports showing that senescent BJ fibroblasts and MEFs can be stimulated to divide upon disruption of the p53 pathway, whereas WI38 cells cannot (Dirac and Bernards, 2003; Beauséjour et al., submitted). Furthermore, senescent IMR90 cells do not divide following the introduction of E1A, although quiescent IMR90 cells readily do so (S.N. and S.W.L., unpublished). Together, these results link SAHFs to an essentially irre-

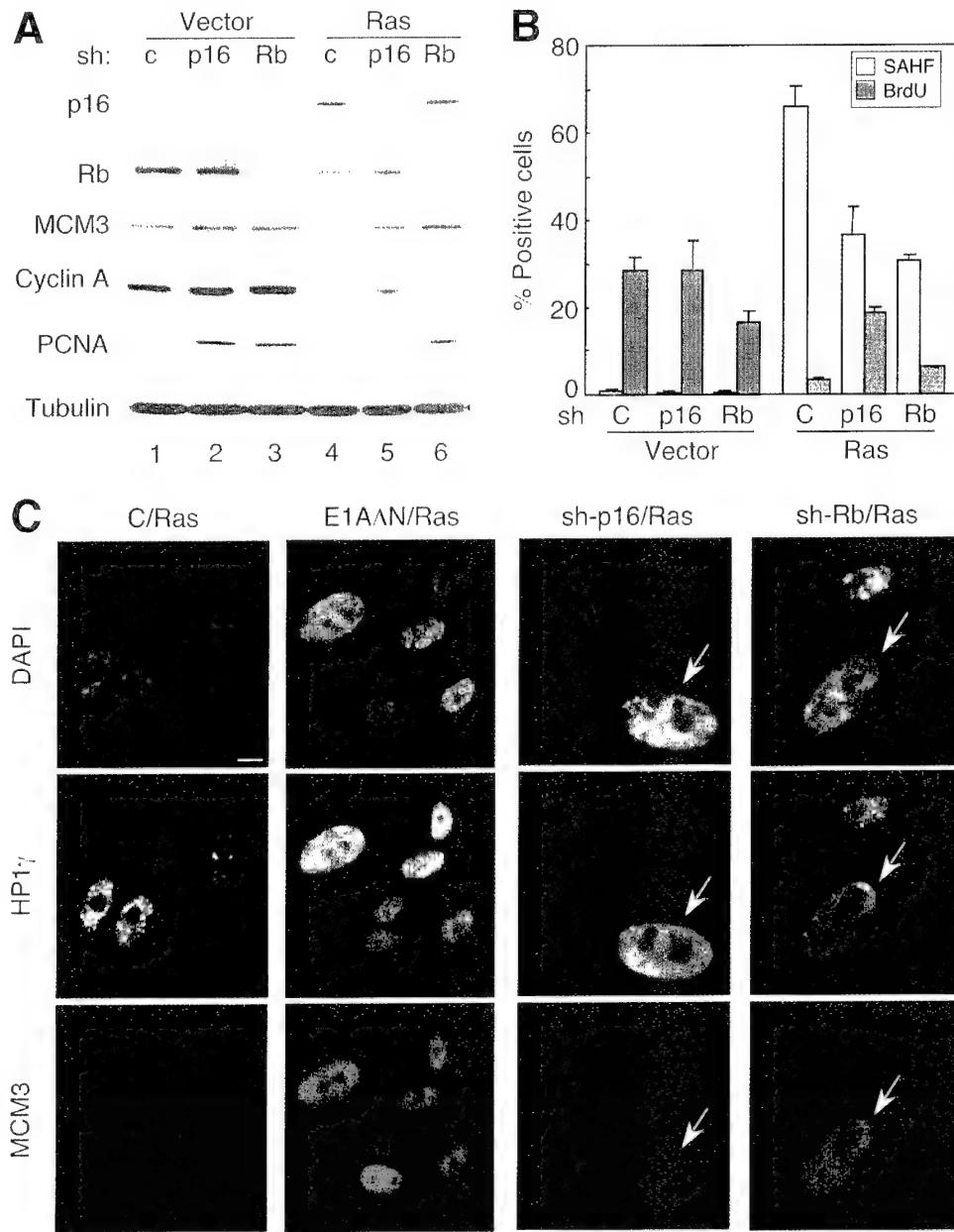


Figure 7. The p16^{INK4a}/Rb Pathway Is Crucial for SAHF Formation and Gene Silencing

(A) The expression of the indicated protein was determined by immunoblotting of extracts from IMR90 cells expressing empty vector or H-rasV12 (Ras) in combination with short hairpin RNAs (sh) against p16^{INK4a} or Rb, as well as control vector (c) at day 5 (see Figure 1B).

(B) The cells were also assessed for SAHF formation and BrdU incorporation at the same time point as in (A).

(C) Indirect immunofluorescent images for HP1 γ and MCM3 of IMR90 cells expressing the indicated genes. Cells were at PS day 8 (PS8) (see Figure 1B). The nuclei, which failed to form SAHFs in sh-p16/Ras and sh-Rb/Ras, are indicated with arrows. DNA was counterstained by DAPI. Scale bar to 10 μ m.

versible cell cycle arrest and imply that, in the absence of SAHF formation, this arrest is more difficult to maintain.

Quiescence Versus Senescence

Much of what we know concerning the regulation of E2F activity comes from studies examining cell cycle transitions into and out of a quiescent state (Trimarchi and Lees, 2002). These transitions are controlled in a reversible manner, in part, by the competing action of HATs and HDACs on the histones of E2F target promot-

ers (see Introduction). In this study, we compared the physical state and regulation of E2F target genes in quiescent and senescent cells. In both cell states, the amount of K9-acetylated histone H3 that associates with E2F target promoters declines, consistent with the downregulation of transcription that accompanies cell cycle exit. However, in senescent IMR90 cells, histone H3 acetylation is ultimately replaced by methylation at lysine 9, an apparently irreversible modification that prevents acetylation by HATs and is barely observed on

E2F-responsive promoters in quiescent cells (see Figure 4C). Methylated lysine 9 forms a docking site for HP1 proteins and, accordingly, HP1 γ preferentially associates with E2F target promoters in senescent cells. These modifications are predicted to form a "lock" on the transcription of E2F responsive promoters, making them less accessible to the transcription machinery (Dillon and Festenstein, 2002; Trimarchi and Lees, 2002). Accordingly, several E2F-responsive genes in senescent cells are stably repressed and insensitive to enforced E2F expression relative to quiescent cells (see Figure 5). Although it remains to be determined whether every E2F target gene behaves as those studied here, their transition to a heterochromatin-like organization may contribute to the insensitivity of senescent cells to mitogenic signals and the apparent irreversibility of the senescence process.

What determines whether a cell enters quiescence or senescence? Although many factors undoubtedly play a role, our results imply that Rb is crucial for this decision. Interestingly, although the current paradigm for Rb action suggests that Rb negatively regulates normal cell cycle transitions by recruiting HDACs to transcriptionally repress E2F target genes, recent reports have been unable to detect Rb on E2F target promoters in G1 and quiescent cells (Rayman et al., 2002; Takahashi et al., 2000). In contrast, p107 and p130 were readily detected. While we confirm these reports, we also show that Rb appears on some E2F target promoters in senescent cells. Since Rb can associate with certain histone methyltransferases and the HP1 proteins (Nielsen et al., 2001b), the simplest model to explain our results is that Rb acts directly on E2F target promoters to nucleate regions of heterochromatin leading to the silencing of E2F target genes and perhaps spreading to other euchromatic loci. However, we cannot exclude the possibility that Rb acts at other sites in the genome or controls the process indirectly, perhaps by influencing the expression or activity of chromatin remodeling factors.

Our model implies that the decision to enter senescence is determined, in part, by a histone methyltransferase (HMT) that acts with Rb and HP1 proteins to alter chromatin structure and silence E2F target genes. One candidate for this molecule is SUV39H1, which is a known regulator of PEV in *Drosophila* and required for the maintenance of pericentric heterochromatin in mammalian cells (Peters et al., 2001). Consistent with this possibility, SUV39H1 can interact with Rb and affect the expression of some E2F target genes (Nielsen et al., 2001b). Moreover, enforced expression of SUV39H1 induces a senescent-like arrest in IMR90 fibroblasts in a manner that depends on its methyltransferase activity (M.N. and S.W.L., unpublished data). However, Rb can interact with other histone methyltransferases (Steele-Perkins et al., 2001), and Suv39h is not required for all types of heterochromatin formation (Peters et al., 2002). Furthermore, *Suv39h1/h2*-deficient MEFs arrest in response to oncogenic *ras* (M.N., T. Jenuwein, and S.W.L., unpublished data). Therefore, another HMT may act alone or together with SUV39H1 to mediate SAHF formation and E2F gene silencing during senescence. Presumably these interactions do not occur as cells exit the cell cycle into a quiescent state.

Senescence, Rb, and Tumor Suppression

A large body of work implicates the p53 and Rb pathways in cellular senescence and, as such, the process may function as a natural brake to tumor development. In this study, we show that Rb contributes to senescence by promoting SAHF formation and silencing E2F target genes. Interestingly, in IMR90 cells, Rb is not essential for all aspects of the senescence program, including some of the morphological changes, the accumulation of SA- β -gal activity, and the ultimate exit from the cell cycle. We have proposed that senescence in human diploid fibroblasts involves the combined activities of the p16/Rb pathway, p53, and the promyelocytic leukemia protein (PML) (Ferbeyre et al., 2000), and perhaps p53, PML, or some other activity controls aspects of the senescent morphology and the initial cell cycle exit. However, loss of *INK4a* alone appears sufficient to override senescence in some human fibroblast strains (Brookes et al., 2002), and IMR90 cells with defects in the Rb pathway show a delay in cell cycle exit following expression of oncogenic *ras* and entirely fail to senesce in response to p16^{INK4a}.

Presumably, the failure to silence E2F target genes reduces the probability that a damaged cell undergoes senescence or, alternatively, makes the arrest more difficult to sustain. Consistent with this view, a critical difference between IMR90 and WI38 fibroblasts (which form SAHFs) and BJ fibroblasts and MEFs (which apparently do not) appears to be the relative importance of the p16-Rb pathway in controlling the arrest. Hence, IMR90 and WI38 cells have a robust p16^{INK4a} response during senescence (e.g., Serrano et al., 1997), and studies using RNA interference indicate that p16 is responsible for their inability to cycle upon p53 inactivation (Beauséjour et al., submitted). In contrast, p16^{INK4a} is poorly induced in senescent BJ fibroblasts and appears dispensable for senescence in MEFs (Beauséjour et al., submitted; Lowe and Sherr, 2003). Our results provide a potential mechanism for these observations, implying that p16^{INK4a} upregulation during senescence engages the Rb pathway to produce a permanent arrest by altering the chromatin state of growth regulatory genes. The failure of these processes in cells sustaining *INK4a* or *Rb* mutations may lead to cancer progression or increase the likelihood of tumor relapse from a dormant state.

Mutations arising in malignant tumors often pinpoint processes that must be altered during tumor evolution. Although cellular senescence provides an important brake to human cell transformation in cell culture (Hahn and Weinberg, 2002), its contribution to tumor suppression *in vivo* is poorly defined. Nevertheless, the role of the p16-Rb pathway in cellular senescence coupled with its frequent mutation in human cancers provides strong circumstantial evidence that the senescence program limits the development of malignant tumors. Moreover, many anticancer agents can induce cellular senescence, and the p16^{INK4a}/Rb pathway is important for drug-induced senescence *in vivo* (Chang et al., 1999; Schmitt et al., 2002; te Poele et al., 2002). Our results provide some of the first mechanistic insights into the effector mechanisms of senescence and, as such, may identify processes that control cancer progression and whether tumors undergo a sustained response to therapy.

Experimental Procedures**Retroviral Vectors**

The following retroviral vectors were used in this study: pBabe-Puro (Oncogenic *ras* (H-RasV12) and *MEK1* Q56P) (Lin et al., 1998); pWZL Hygro (H-RasV12, *p16^{INK4a}*, and human *p53^{175H}*) (Serrano et al., 1997); pLPC-Puro (EGFP-tagged human *HP1 β* cDNA and *E1A* and *E1A* Δ N) (Samuelson and Lowe, 1997); and pMSCV-puro (Clontech) (*INK4a* and *Rb* shRNAs). Additional information can be found in the Supplemental Data available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>.

Cell Culture and Gene Transfer

Human diploid IMR90 fibroblasts (ATCC) were cultured in DMEM supplemented with 10% FBS and antibiotics. Retroviruses were packed using the Phoenix cells (G. Nolan, Stanford University, CA) and infections were performed as described (Serrano et al., 1997) except that amphotropic viruses were used to produce cells for the ChIP assays and RNAi experiments. The infected population was selected using either 2 μ g/ml puromycin (Sigma) for 2–3 days or 100 μ g/ml hygromycin B (Roche) for 2–3 days. For coinfection of puromycin- and hygromycin-selectable vectors, cells were selected with puromycin first for 2 days, followed by hygromycin selection for another 2 days. Adenoviruses were introduced into quiescent and senescent IMR90 cells at a multiplicity of infection (MOI) of 15 plaque-forming units (PFU) cell $^{-1}$. For the experiments in Figure 5B, Ad-GFP was used at an MOI of 4 PFU cell $^{-1}$ and Ad-E2F1 was used in a range of 0.03 and 15 PFU cell $^{-1}$.

Cell Proliferation and SA- β -gal Assays

IMR90 cells were plated on coverslips and subsequently labeled with 5-Bromo-2'-deoxyuridine (BrdU, 100 μ g/ml, Sigma) and 5-fluor-2'-deoxyuridine (FdU, 10 mg/ml, Sigma) for 6 hr. Nuclei incorporating BrdU were visualized by immunolabeling using anti-BrdU antibody (Pharmingen, 1:400) as previously described (Humbert et al., 2000). SA- β -gal activity was detected as previously described (Serrano et al., 1997). DNA was visualized by DAPI (1 μ g/ml) after permeabilization with 0.2% Triton X-100/PBS.

Immunolabeling, Electron Microscopy, and RNA FISH

Immunofluorescence studies were performed using standard procedures (see Supplemental Data available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>) using the following primary antibodies: anti-*HP1 α* antibody (1:200, provided by W.C. Earnshaw); anti-*HP1 β* antibody (2MOD-1A9, 1:500, provided by P. Chambon); anti-*HP1 γ* antibody (2MOD-1G6, 1:5000, provided by P. Chambon); anti-*K9*, 14Ac-H3 antibody (Upstate, 1:1000); anti-*K9M-H3* antibody (provided by C.D. Allis and Upstate, 1:1500); anti-*K4M-H3* antibody (Upstate, 1:700); anti-*MCM3* antibody (provided by B. Stillman, 1:200); and anti-*Rb* antibody (G3-245, Pharmingen, 1:100) together with XZ-55 and C36 hybridoma supernatant (1:50).

Electron microscopy was performed as previously described (Woo et al., 1998). For DNA/RNA labeling, IMR90 cells, cultured on Thermanox (Electron Microscopy Sciences) coverslips, were fixed with 1.5% glutaraldehyde in PBS, ethanol dehydrated at 0 to minus 20°C, embedded in Lowicryl K4M resin (Electron Microscopy Sciences) and polymerized at -36° C. Thin sections cut en face at 100 nm thickness were collected on uncoated nickel grids, incubated in essentially globulin-free 1% BSA (Sigma) in PBS, incubated in drops of primary mouse monoclonal antibody to DNA (AC-30-10 Maine Biotechnology Services) diluted in PBS, for one hour at 22°C and then rinsed in PBS and incubated for 30 min in drops of secondary antibodies conjugated to 10 nm colloidal gold (Pharmacia). After immunogold labeling, sections were rinsed in distilled water, air dried and briefly counterstained with 3% aqueous uranyl acetate. RNase-gold solution was freshly prepared by conjugating RNase T₁ from *Aspergillus oryzae* (Sigma) to 20 nm colloidal gold particles (Chencllet and Bendayan, 1990). Sections were incubated in RNase gold for 15 min at 37°C, rinsed, and stained as above.

RNA FISH was performed as described elsewhere (Clemson et al., 1996) (see Supplemental Data available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>) using bacterial artificial chromo-

some (BAC) clones CTD-2097K16 and CTD-2217D23 (ResGen) as probes for *INK4a* and *cyclin A* genes, respectively.

Micrococcal Nuclease Assay

Cells were permeabilized with 0.01% L- α -lysophosphatidylcholine (Sigma) in 150 mM sucrose, 80 mM KCl, 35 mM HEPES [pH 7.4], 5 mM K₂HPO₄, 5 mM MgCl₂, and 0.5 mM CaCl₂ for 90 s, followed by digestion with 2 U/ml micrococcal nuclease (Sigma) in 20 mM sucrose, 50 mM Tris [pH 7.5], 50 mM NaCl, and 2 mM CaCl₂ at room temperature for various times. DNA was isolated and subjected to 0.8% agarose gel electrophoresis.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitations were performed as described previously (Nahle et al., 2002) using anti-*K9/14Ac-H3* (Upstate), *K9M-H3* (ab7312, Abcam, Upstate, or provided by C.D. Allis), *HP1 γ* (Chemicon), *Rb* (C-15, Santa Cruz), *p107* (C-18, Santa Cruz), and *p130* (C-20, Santa Cruz) antibodies. DNA released from precipitated complexes was amplified using sequence specific primers by PCR. PCR products were labeled by [α -³²P]dCTP (Amersham Pharmacia), separated on a 4% nondenaturing polyacrylamide gel. The primer sets used were: promoter regions of *cyclin A*, *PCNA*, *p16^{INK4a}*, *stromelysin-1*, and β -*actin* (sequences are available from the authors upon request).

Western Blotting

Western blotting analysis was carried out on 20 μ g whole-cell lysate by using enhanced chemiluminescence (ECL; Amersham) detection as previously described (Serrano et al., 1997). Blots were probed with the following antibodies: anti-*p16* antibody (NA29, Oncogene, 1: 200); anti-*Ras* antibody (OP23, Oncogene, 1: 300); anti-*Rb* antibody (G3-245, Pharmingen, 1:1000) together with XZ-55 hybridoma supernatant (1:100); anti-*E2F1* antibody (KH95, Santa Cruz, 1: 200); anti-*cyclin A* antibody (Sigma, 1:1000); anti-*PCNA* antibody (PC10 hybridoma supernatant, 1:1000); anti-*MCM3* antibody (provided by B. Stillman, 1:500); anti-*E1A* antibody (M73 hybridoma supernatant, 1: 300); anti-*p53* (DO1, Oncogene, 1:1000); and anti- α -tubulin (B-5-1-2, Sigma, 1: 2000).

Real-Time PCR

Total RNA was isolated by RNeasy Mini Kit (Qiagen) and was converted to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems). Gene-specific TaqMan primer sets were designed using Primer Express 1.5 (sequences are available from the authors upon request). Real-time PCR was carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7700 Sequence Detector (Applied Biosystems) and β -actin serves as an endogenous normalization control. Sequence Detector software (version 1.7) was utilized for data analysis and relative fold induction was determined by the comparative threshold cycle method.

Acknowledgments

We thank J.R. Nevins and K. Helin for E2F-1 expression vectors; W.C. Earnshaw, P. Chambon, C.D. Allis, and B. Stillman for antibodies; T. Howard for electron microscopy; M. McCurrach for editorial advice; and J. Duffy and C. Eberstark for help in preparing the figures. We also thank T. Jenuwein, S. Grewal, R. Martienssen, and members of the Lowe laboratory for stimulating discussions and helpful advice. This work was supported by DOD-BCRP postdoctoral fellowship (DAMD17-01-1-0209) and Uehara Memorial Foundation research fellowship (M.N.), and grants GM42694, CA13106, and AG16379 (D.L.S., G.J.H., S.W.L.), from the National Institutes of Health, and a generous gift from the Ann L. and Herbert J. Siegel Philanthropic Fund.

Received: December 9, 2002

Revised: May 13, 2003

Accepted: May 16, 2003

Published: June 12, 2003

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Reversal of human cellular senescence: roles of the p53 and p16 pathways

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Telomere erosion and subsequent dysfunction limits the proliferation of normal human cells by a process termed replicative senescence. Replicative senescence is thought to suppress tumorigenesis by establishing an essentially irreversible growth arrest that requires activities of the p53 and pRB tumor suppressor proteins. We show that, depending on expression of the pRB regulator p16, replicative senescence is not necessarily irreversible. We used lentiviruses to express specific viral and cellular proteins in senescent human fibroblasts and mammary epithelial cells. Expression of telomerase did not reverse the senescence arrest. However, cells with low levels of p16 at senescence resumed robust growth upon p53 inactivation, and limited growth upon expression of oncogenic RAS. In contrast, cells with high levels of p16 at senescence failed to proliferate upon p53 inactivation or RAS expression, although they re-entered the cell cycle without growth after pRB inactivation. Our results indicate that the senescence response to telomere dysfunction is reversible and is maintained primarily by p53. However, p16 provides a dominant second barrier to the unlimited growth of human cells.

Keywords: cyclin-dependent kinase/pRB/RAS/senescence/telomerase

Introduction

Normal cells do not divide indefinitely due to a process termed replicative senescence. One important mechanism responsible for the replicative senescence of human cells is the erosion and eventual dysfunction of telomeres (Harley *et al.*, 1990; de Lange, 2001). Telomeres are the DNA sequence and associated proteins that cap and stabilize the ends of linear chromosomes, preventing their degradation or fusion by DNA repair systems. Owing to the biochemistry of DNA replication, several dozen base pairs of telomeric DNA are lost with each cell cycle. Thus, proliferating cells experience progressive telomere short-

ening, unless they express the enzyme telomerase, which can add the telomeric sequence to chromosome ends *de novo*. Most human cells do not express this enzyme, and hence can acquire telomeres that are critically short and dysfunctional. Dysfunctional telomeres signal normal cells to cease proliferation with a characteristic senescent phenotype (Blackburn, 2001; Shay and Wright, 2001; Kim *et al.*, 2002).

Relicative senescence is an example of a more general process, herein termed cellular senescence, which arrests the growth of cells in response to many stimuli. These stimuli include dysfunctional telomeres, DNA damage, disrupted chromatin organization, and certain oncogenes, such as activated RAS (Campisi *et al.*, 2001; Serrano and Blasco, 2001). They have in common the potential to initiate or promote neoplastic transformation. Cellular and replicative senescence require activities of the p53 and pRB tumor suppressor proteins, which regulate pathways that suffer mutations in most, if not all, mammalian cancers. Human cells that lose p53 and pRB function are generally refractory to multiple senescence-inducing stimuli (Serrano *et al.*, 1997; Dimri *et al.*, 2000). These and other lines of evidence suggest that the senescence response suppresses the development of cancer in mammals (Reddel, 2000; Campisi *et al.*, 2001; Wright and Shay, 2001).

Although p53 and pRB are clearly critical for establishing the senescence growth arrest, their precise roles in this process are incompletely understood. p53 is presumed to sense dysfunctional telomeres as damaged DNA, whereupon it elicits the senescence response at least in part by increasing expression of the p21 cyclin-dependent kinase inhibitor (CDKI); p21 in turn prevents the phosphorylation and inactivation of pRB (Sherr and Roberts, 1999). However, inactivation of either p53 or pRB (e.g. by viral oncoproteins or anti-sense oligonucleotides) independently extends the replicative lifespan of many human cells, allowing them to proliferate despite short telomeres (Hara *et al.*, 1991; Shay *et al.*, 1991). Thus, although the p53 and pRB pathways interact, they may also act separately to establish the senescence arrest. Indeed, senescent cells have been reported to upregulate another CDKI, p16, which also controls pRB activity (Alcorta *et al.*, 1996; Hara *et al.*, 1996a; Stein *et al.*, 1999). p16 may limit cell proliferation by a mechanism distinct from that utilized by p53, since some human epithelial cells (e.g. initial outgrowths from mammary tissue explants) senesce with relatively long telomeres and high p16 expression (Kiyono *et al.*, 1998; Ramirez *et al.*, 2001). Moreover, ectopic expression of telomerase does not protect such cells from replicative senescence, suggesting that p16 expression and function are independent of telomere status (Kiyono *et al.*, 1998; Rheinwald *et al.*, 2002).

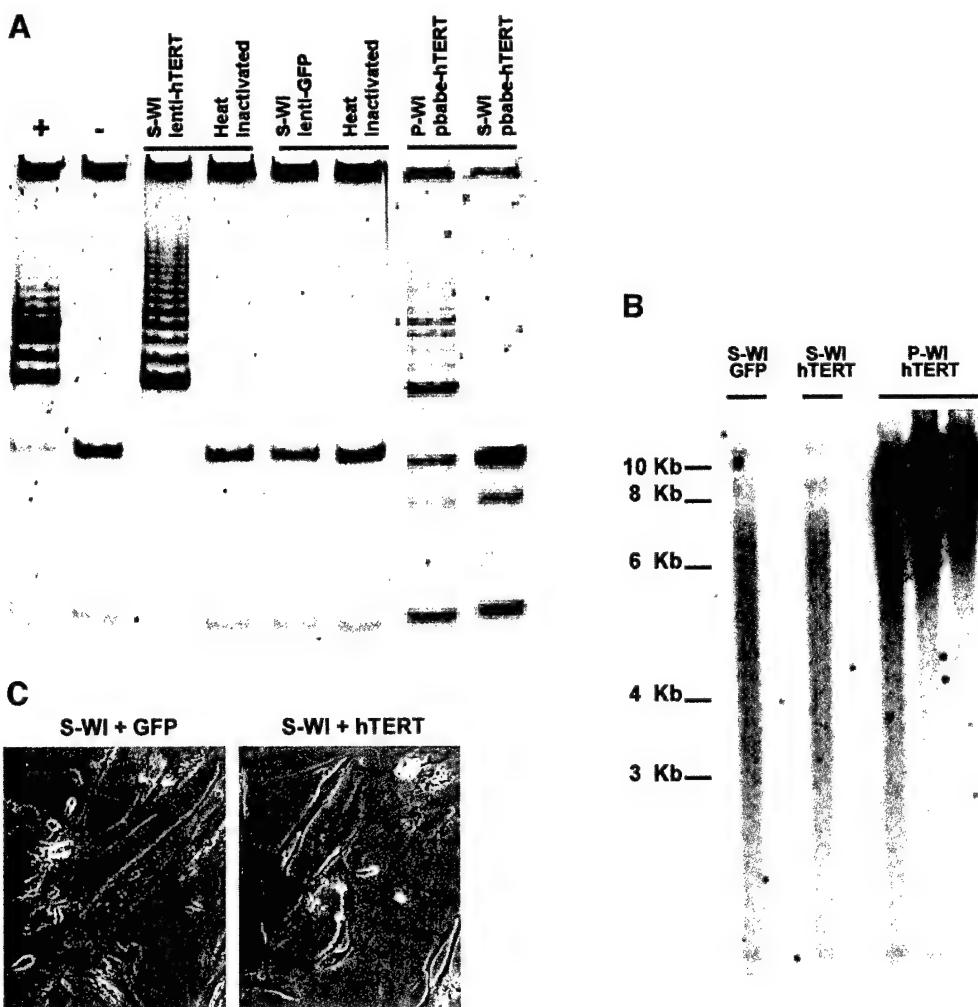


Fig. 1. hTERT does not reverse the senescent phenotype. (A) Lenti-hTERT confers telomerase activity. Senescent (S) or pre-senescent (P) WI-38 (WI) cells were infected with lenti-hTERT, lenti-GFP or pBABE-hTERT, and telomerase activity was measured using the TRAP assay, as described in Materials and methods. '+' is a positive TRAP control, and '-' and 'Heat inactivated' are negative controls. (B) Lenti-hTERT alters telomere length in pre-senescent, but not senescent cells. Terminal restriction fragment (TRF) lengths in P- or S-WI cells infected with lenti-GFP (GFP) or lenti-hTERT (hTERT) were determined as described in Materials and methods. (C) Lenti-hTERT does not alter senescent morphology. S-WI cells were infected with lenti-GFP (+GFP) or lenti-hTERT (+hTERT), and viewed and photographed 7 days later.

p53 and pRB are also important for maintaining the senescence growth arrest, which, in human cells, is thought to be irreversible. Senescent human cells arrest growth with a G₁ DNA content, and cannot be stimulated to divide by physiological mitogens. Moreover, although the potent viral oncogene SV-40 T-antigen stimulates DNA replication in senescent human fibroblasts, it does not stimulate cell proliferation (Ide *et al.*, 1983; Gorman and Cristofalo, 1985). T-antigen binds and inactivates both p53 and pRB, and mutants defective in either p53 or pRB binding fail to stimulate DNA synthesis in senescent cells (Sakamoto *et al.*, 1993; Hara *et al.*, 1996b). One interpretation of these findings is that p53 and pRB cooperatively prevent senescent cells from initiating S phase, but another activity prevents completion of the cell cycle. On the other hand, p53 antibodies, when micro-injected into senescent cells, were shown to stimulate DNA synthesis and limited proliferation (Gire and Wynford-Thomas, 1998). Thus, requirements for main-

taining the senescence arrest are less clear than the requirements for establishing it.

We recently showed that human fibroblasts differ in their sensitivity to BMI-1, an oncogene that extends the replicative lifespan of fibroblasts by repressing p16, apparently because they differ in the level of p16 they express at senescence (Itahana *et al.*, 2003). This finding raises the possibility that human cell strains also differ in the mechanisms that maintain the senescence state. To explore this possibility, and understand the mechanisms that maintain the senescence arrest, we used lentiviruses to express viral and cellular proteins in replicatively senescent human fibroblasts. Our results suggest that the senescence arrest caused by telomere dysfunction is reversible, being maintained primarily by p53 and reversed by p53 inactivation. In some human cells, however, p16 provides a dominant, apparently irreversible, second barrier to cell proliferation, which cannot be completely overcome by subsequent inactivation of pRB.

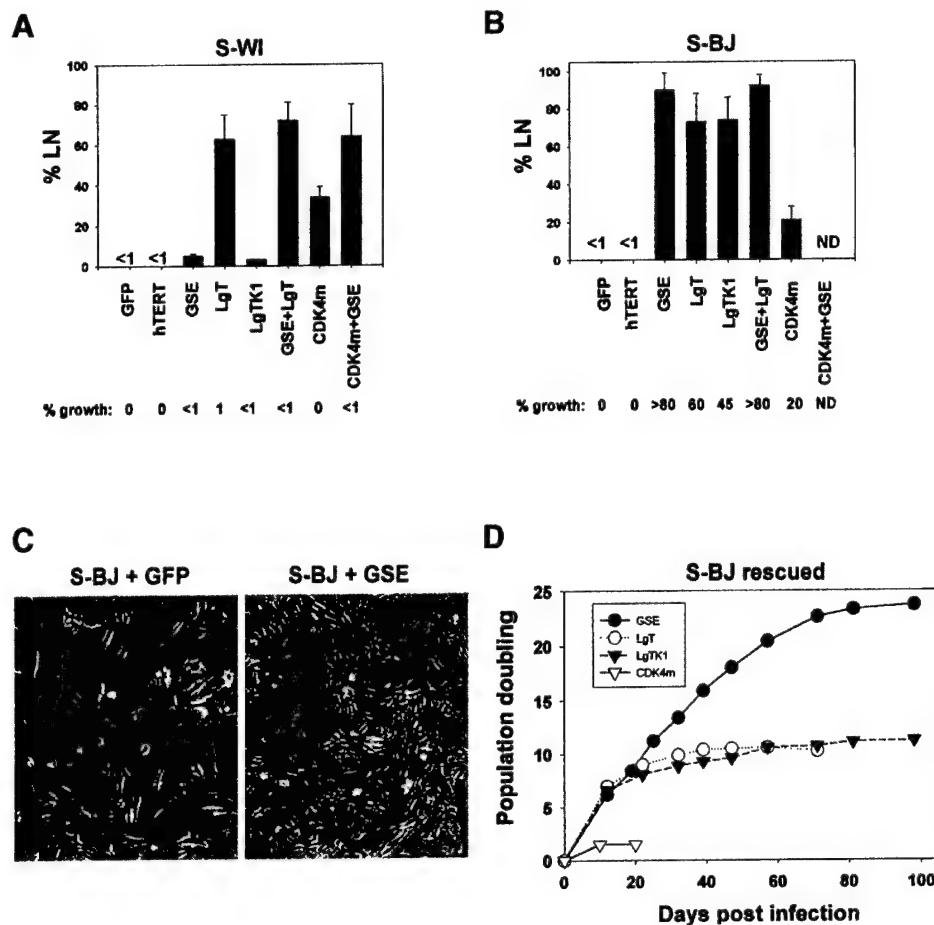


Fig. 2. p53 inactivation reverses senescence of BJ, but not WI-38 fibroblasts. (A) S-WI cells synthesize DNA, but do not proliferate. S-WI cells were infected with lentiviruses expressing GFP, hTERT, GSE, LgT, LgTK1 and CDK4m as indicated; 72 h later, DNA synthesis was determined by % LN, and percentage growth monitored, as described in the text. (B) S-BJ cells synthesize DNA and proliferate. S-BJ cells were infected and monitored, as described in (A). (C) Morphology of control and rescued S-BJ cells. S-BJ cells infected with GFP or GSE-expressing lentivirus were photographed 6 days later. (D) Lifespan assays. S-BJ cells were infected with lentiviruses expressing the indicated proteins, serially passaged, and cell number determined at each passage, as described in Materials and methods.

Results

Telomerase does not reverse the senescence growth arrest

The first candidate we tested for ability to reverse the senescence growth arrest was hTERT, the catalytic subunit and rate-limiting component of telomerase (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). For these and subsequent experiments, we used two human fibroblast strains: WI-38 (WI) from fetal lung and BJ (from neonatal foreskin). Neither strain expresses the endogenous *TERT* gene, and both are devoid of detectable telomerase activity, as determined by the telomere repeat amplification protocol (TRAP) assay.

We passaged pre-senescent (early passage) cultures (P-WI, P-BJ) until replicative senescence. Unless noted otherwise, senescent cultures (S-WI, S-BJ) contained >99.9% non-dividing cells, as determined by no increase in cell number over >4 wks and <1% [³H]thymidine-labeled nuclei after a 3-day labeling interval (% LN). To express hTERT and other proteins, we used lentiviruses, which efficiently infect and stably express genes in non-

dividing cells (Bukrinsky *et al.*, 1993). We verified the infection efficiency by infecting parallel cultures with an equivalent titer (see Materials and methods) of virus expressing green fluorescent protein (GFP), and, where possible, immunostaining for the virally expressed proteins. At the titers employed, the lentiviruses transduced >95% of cells in both pre-senescent and senescent cultures.

The hTERT-expressing lentivirus (lenti-hTERT) conferred robust telomerase activity on S-WI cells, whereas S-WI cells infected with lenti-GFP were devoid of telomerase activity (Figure 1A). By contrast, pBabe-hTERT, which requires cell proliferation for integration and expression, failed to confer telomerase activity on S-WI cells, although it conferred robust activity on P-WI cells (Figure 1A).

S-WI cells infected with lenti-hTERT did not proliferate (Figure 2A), despite expressing high levels of telomerase. Moreover, they did not lose the senescent morphology (Figure 1C) or senescence-associated β -galactosidase (SA-Bgal) expression (Dimri *et al.*, 1995) (not shown). Identical results were obtained when S-BJ cells were

infected with lenti-hTERT (Figure 2B, and results not shown). Lenti-hTERT did not alter average telomere length in S-WI cells, even when tested up to 6 weeks after infection; however, the same virus elongated telomeres in P-WI cells (Figure 1B), indicating that the virus expressed a functional hTERT protein.

These results lead to two important conclusions. First, human telomeres cannot be modified by telomerase in the absence of cell proliferation. Secondly, telomerase cannot reverse the growth arrest or senescent phenotype of replicatively senescent cells.

p53 inactivation reverses senescence of BJ, but not WI-38, cells

Inactivation of either the p53 or pRB pathway is known to postpone, but not prevent, the replicative senescence of human fibroblasts (Wright and Shay, 2001). Inactivation of both pathways, for example by SV-40 T-antigen (LgT), causes crisis, a state characterized by genomic instability, cell death, and the eventual emergence of rare replicatively immortal variants (Shay *et al.*, 1993; Wei and Sedivy, 1999).

To explore the roles of p53 and pRB in maintaining the senescence arrest, we used lentiviruses to express the following proteins in human cells: (i) LgT, which binds and inactivates both p53 and pRb (Fanning, 1992); (ii) LgT-K1, a LgT mutant that binds p53 but not pRb or pRB family members (DeCaprio *et al.*, 1988); (iii) GSE-22, a peptide that inactivates p53 function in a dominant negative fashion (Gudkov *et al.*, 1993); and (iv) CDK4m, a cyclin-dependent kinase 4 mutant that cannot bind p16, and hence constitutively inactivates pRB (Wolfel *et al.*, 1995), although it may also have other modes of action.

Consistent with results from SV-40 infection and plasmid microinjection experiments (Gorman and Cristofalo, 1985; Sakamoto *et al.*, 1993; Hara *et al.*, 1996b) (Figure 2A), LgT stimulated a substantial fraction (60–70%) of S-WI cells to synthesize DNA. CDK4m also stimulated DNA synthesis in S-WI, albeit to a lesser extent (35–40%). By contrast, GSE-22 and LgT-K1 were essentially inactive (<5%). These data suggest that S-WI cells can re-enter the cell cycle upon inactivation of the pRB pathway (by LgT or CDK4m), but inactivation of the p53 pathway alone (by LgT-K1 or GSE) has no effect in these cells. However, regardless of ability to stimulate DNA synthesis, none of the lentiviruses, alone or in combination, efficiently stimulated S-WI cells to proliferate (Figure 2A). We conclude that although S-WI cells enter S-phase upon inactivation of the pRB pathway, they cannot complete the cell cycle and proliferate.

In contrast, a substantial fraction of S-BJ cells initiated DNA synthesis in response to each of the four lentiviruses expressed proteins (LgT, LgT-K1, GSE-22 and CDK4m) (Figure 2B). Moreover, GSE and LgT-K1 were as effective as LgT, each stimulating 70–90% of the cells (Figure 2B). CDK4m was less effective (25–30%) (Figure 2B). Most striking, all four lentiviruses each stimulated S-BJ cells to complete the cell cycle and proliferate. The extent of proliferation was approximately equal to the extent of DNA synthesis. Proliferation was assessed by the formation of colonies (>50 cells) (Figure 2B) and loss of senescent morphology

(Figure 2C). Thus, in contrast to S-WI cells, the growth arrest of replicatively senescent BJ fibroblasts was completely reversible, and p53 inactivation was sufficient to induce both DNA synthesis and proliferation.

With regard to efficacy, GSE-22 was more efficient than LgT or LgT-K1 at reversing the senescence arrest of S-BJ cells (Figure 2B), consistent with reports that LgT and LgT-K1 do not completely inactivate p53 (Deppert *et al.*, 1987). Moreover, failure of GSE-22 to stimulate S-WI cells was not due to an inability to inactivate p53 in these cells. GSE-22 increased p53 levels in both S-BJ and S-WI cells, as expected from its ability to enhance p53 stabilization (Gudkov *et al.*, 1993); moreover, GSE-22 markedly reduced p21 levels in both cell strains, as expected for loss of p53 function (Supplementary figure 1, available at *The EMBO Journal* Online). Interestingly, CDK4m stimulated DNA synthesis in both S-WI and S-BJ cells (25–35%), despite their different requirements for cell cycle re-entry after senescence. CDK4m may also act indirectly on the p53 block by sequestering p21 (Sherr and Roberts, 1999). Consistent with p21 sequestration accounting for the ability of CDK4m to stimulate S-BJ cells, most of the p21 in S-BJ cells co-immunoprecipitated with CDK4 after infection with lenti-CDK4m (Supplementary figure 2).

To determine the extent to which GSE, LgT, LgT-K1 and CDK4m stimulated S-BJ cell proliferation, we determined the growth of mass cultures after infection. GSE stimulated >20 additional population doublings (PDs) (Figure 2D). Towards the end of this extended growth, the fraction of cells that synthesized DNA (% LN) gradually declined (not shown). However, the % LN did not decline below 20–25%, even after there was no net increase in cell number. This phenotype (high labeling index without net proliferation) is characteristic of cultures in crisis (Wei and Sedivy, 1999). LgT and LgT-K1 each stimulated 10–11 additional PDs (Figure 2D), also culminating in crisis. Cultures driven to crisis by GSE-22 showed fewer apoptotic cells than cultures driven by LgT or LgT-K1 (not shown), which likely explains why GSE-22-expressing cultures completed more PDs than LgT- or LgT-K1-expressing cultures. In contrast to the other proteins, CDK4m stimulated very limited proliferation of S-BJ cells (2–3 PDs) (Figure 2D). This finding suggests that although p21 binding by CDK4m may partially relieve the p53 block (Figure 2B and Supplementary figure 2), it is not equivalent to p53 inactivation.

Together, the results indicate that p53 maintains the senescence growth arrest of S-BJ cells, and that inactivation of p53 alone is sufficient to reset their replicative lifespan. A third human fibroblast strain (82-6, from skin) displayed an intermediate response to p53 inactivation: 20–25% of senescent 82-6 cells initiated DNA synthesis in response to GSE-22 (not shown). Thus, some human fibroblast strains have phenotypes intermediate between WI-38 and BJ with respect to reversibility of the senescence growth arrest by p53 inactivation.

p16 prevents reversal of senescence by p53 inactivation

Why do S-BJ cells resume growth upon p53 inactivation, without pRB inactivation, whereas S-WI cells fail to proliferate (despite undergoing DNA synthesis) even when

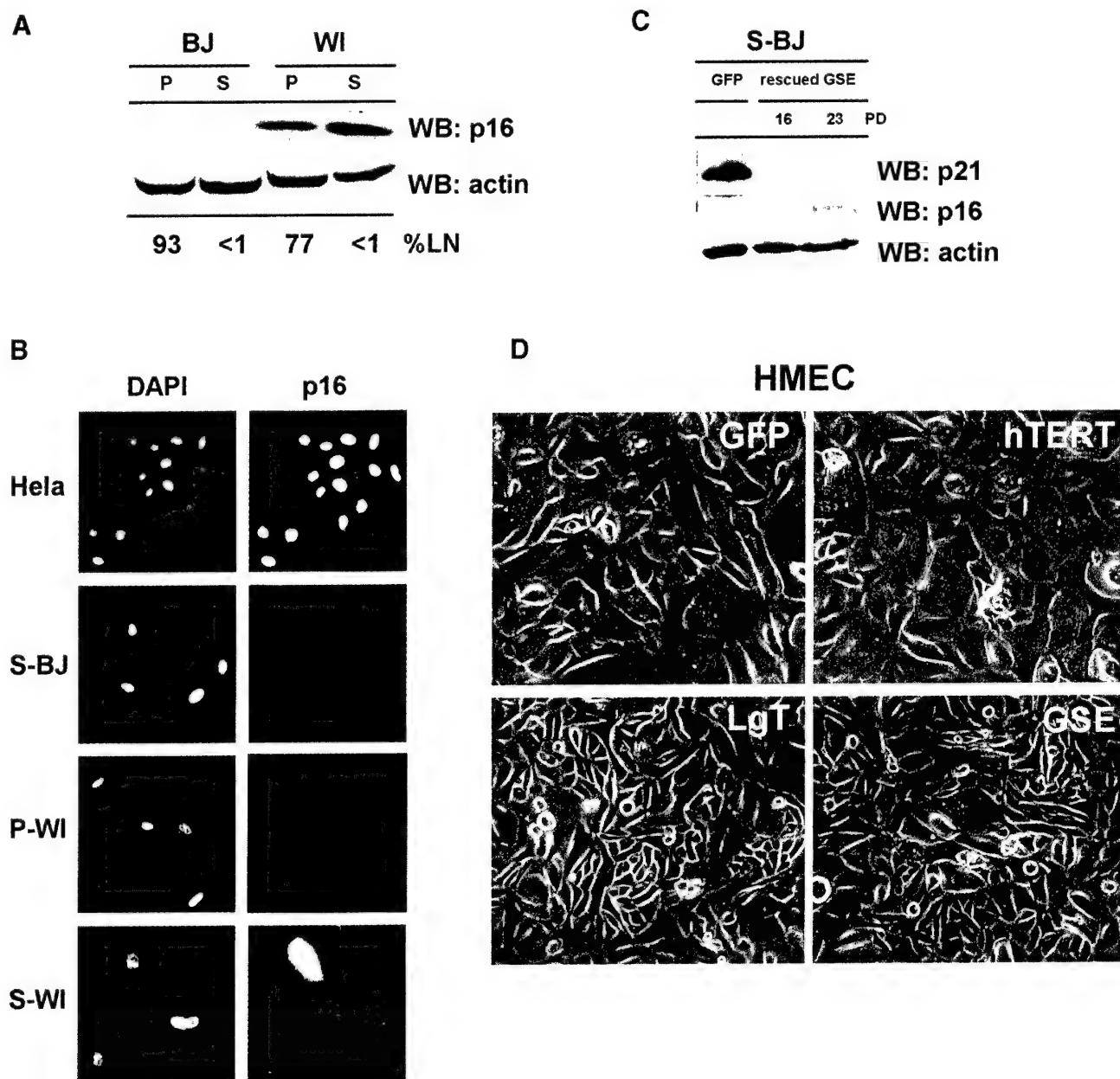


Fig. 3. Senescence reversal correlates inversely with p16 expression. (A) p16 expression. p16 and actin (control) protein levels were assessed in pre-senescent (P) and senescent (S) BJ and WI-38 (WI) cells by western blotting (WB). The labeling index of the cultures is shown below the blot (% LN). (B) p16 immunostaining. Pre-senescent (P) and senescent (S) BJ and WI-38 cells were immunostained for p16, and nuclei stained with DAPI, as described in Materials and methods. HeLa cells served as a positive control. (C) p16 and p21 levels after rescue from senescence by GSE-22. S-BJ cells were infected with control (GFP) or GSE-22-expressing (rescued GSE) lentivirus. Rescued cells were harvested while proliferating (16 PDs) or after proliferation ceased (23 PDs), and analyzed for p21, p16 and actin (control) by western blotting. (D) Senescence reversal in HMECs. Post-selection HMECs were infected with lentiviruses expressing the indicated proteins, and monitored for growth, as described in Materials and methods. Shown are cells 72 h following infection.

both pRB and p53 are inactivated? One possibility might be intrinsic differences in the ability to induce p16 at senescence. WI-38, like several human epithelial cells, appear to undergo replicative senescence prior to critical telomere shortening owing to induction of p16 by as yet unidentified factors (Wright and Shay, 2001; Itahana *et al.*, 2003). Thus, p16 may impose a proliferative block that cannot be overcome by p53 inactivation. Consistent with this idea, WI-38 cells consistently expressed higher levels

of p16 than BJ cells, whether pre-senescent or senescent (Figure 3A and B). Moreover, S-BJ cells that were rescued from senescence by GSE-22 ceased proliferation (after >20 additional PDs; Figure 2D) with low but significant p16 expression (Figure 3C), and could not be rescued from this second growth arrest by LgT (not shown). Finally, senescent 82-6 fibroblasts expressed p16 at levels intermediate between S-WI and S-BJ cells; immediately after rescue by GSE-22, the cells had substantially less p16 than

the starting population (not shown), suggesting that GSE rescued only those cells that expressed little or no p16. Thus, p16 may prevent reversal of the senescence arrest by p53 inactivation.

Results with human mammary epithelial cells (HMECs), a culture system in which p16 expression is well characterized, support this idea. HMECs that proliferate from tissue explants generally do so for only 10–25 PDs before undergoing a senescence arrest with relatively long telomeres and high p16 expression. However, variants that spontaneously silence p16 by methylation can emerge (self selection) (Hammond *et al.*, 1984). Post-selection HMECs proliferate for an additional 50–75 PDs before senescing with short telomeres and genomic instability, a crisis-like state termed agonescence (Romanov *et al.*, 2001). Agonescent HMECs resumed proliferation upon expression of LgT or GSE, but not hTERT (Figure 3D), and thus resembled S-BJ fibroblasts. However, pre-selection HMECs that ceased growth with high p16 levels were not rescued from the growth arrest by LgT (not shown), suggesting that the senescence arrest is reversible only in p16-negative cells.

Suppression of p16 confers sensitivity to senescence reversal by p53 inactivation

To evaluate more critically the role of p16 in the senescence arrest, we manipulated p16 expression in human fibroblasts. First, we used RNA interference (Paddison *et al.*, 2002) to stably suppress p16 expression in WI38 cells. A short hairpin RNA (shRNA) capable of suppressing p16 expression (Narita *et al.*, 2003) was introduced into proliferating (P-WI) cells using a retroviral vector. The p16 shRNA modestly extended the replicative lifespan of P-WI cells (3–4 PDs) (Supplementary figure 3), and the cells senesced with significantly reduced p16 levels (Figure 4A). S-WI cells with suppressed p16 senesced with elevated levels of p21 (Figure 4A), suggesting that they now senesced primarily due to activation of the p53 pathway, similar to the phenotype of BJ fibroblasts. Indeed, subsequent infection with lenti-GSE-22 completely reversed the senescence arrest of p16-suppressed S-WI cells (Figure 4B), stimulating both DNA synthesis and cell proliferation (>70%). Thus, GSE-22 acted similarly in S-BJ and p16-suppressed S-WI cells, in contrast to its effects in S-WI cells that express high p16 (compare Figure 2A and B with Figure 4B). Next, we ectopically expressed p16 in P-WI cells using a lentivirus (lenti-p16). Western blotting (Figure 4C) and immunofluorescence (Supplementary figure 4) showed that lenti-p16 markedly elevated p16 expression in P-WI cells. As expected (McConnell *et al.*, 1998), lenti-p16 infected P-WI cells arrested growth with a senescent morphology and SA-Bgal expression (not shown). Subsequent infection with lenti-GSE-22 failed to stimulate DNA synthesis or cell proliferation (Supplementary figure 5), similar to its effect in S-WI cells.

Taken together, these results support the idea that p16 imposes a senescence-associated block to cell proliferation that cannot be reversed by p53 inactivation.

Sequential action of p16

p16 is known to exert its effects through pRB, specifically by inhibiting CDKs and thus preventing pRB inactivation

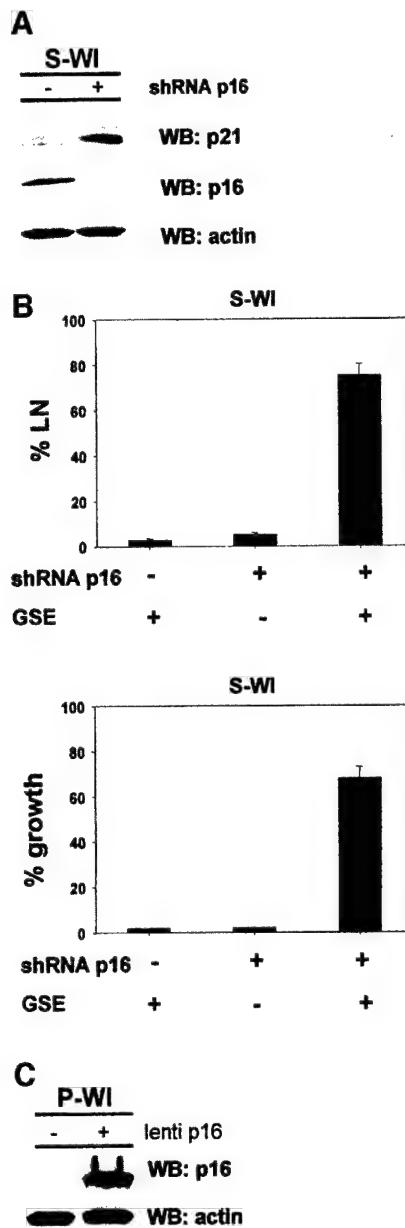


Fig. 4. p16 suppression allows senescence reversal in WI-38 cells. (A) shRNA-p16 suppresses p16 expression in WI-38 cells. P-WI cells were mock infected (–) or infected with shRNA-p16 expressing pMSCV retroviruses, and passaged until replicatively senescent (S-WI; % LN <1%). p16, p21 and actin (control) levels were assessed by western blotting (WB). (B) % LN. P-WI were mock infected (+) and cultured until senescent (S-WI). Cells were then monitored for ability to synthesize DNA (% LN) upon p53 inactivation by subsequent infection with lenti-GSE-22 (GSE). Parallel cultures were monitored for proliferation (% growth) as described in Materials and methods. (C) Ectopic p16 expression. P-WI cells were mock infected (–) or infected (+) with lenti-p16; p16 and actin (control) were assessed by western blotting (WB). At the exposure shown, endogenous p16 in mock-infected P-WI cells is undetectable.

by phosphorylation. However, LgT, which inactivates pRB by direct binding, stimulated DNA synthesis but not proliferation in p16-expressing cells (S-WI or pre-selection HMECs). This finding raises the possibility that the block to cell proliferation imposed by p16 can be independent of continual pRB activity. To explore the

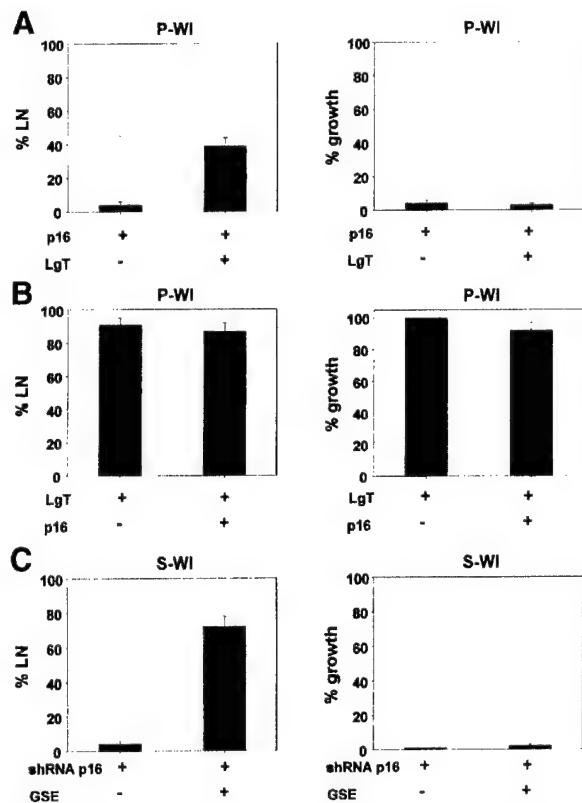


Fig. 5. Sequential inactivation of p16/Rb determines senescence reversibility. (A) p16 followed by LgT. P-WI cells were infected with lenti-p16, and then mock infected (−) or infected with lenti-LgT (+). Cells were monitored for ability to synthesize DNA (% LN) and proliferate (% growth), as described in Materials and methods. (B) LgT followed by p16. P-WI cells were infected with lenti-LgT, followed by mock infection (−) or lenti-p16 infection (+). The infected cells were monitored for DNA synthesis (% LN) and proliferation (% growth). (C) Silencing p16 after senescence. S-WI cells were infected with lenti-shRNA-p16, and then mock infected (−) or infected with lenti-GSE-22 (+). The infected cells were monitored for DNA synthesis (% LN) and proliferation (% growth).

nature of the p16 block, we infected P-WI cells with lenti-p16, and superinfected with lenti-LgT. LgT stimulated DNA synthesis in ~40% of the p16-expressing P-WI cells (Figure 5A), but cell proliferation did not occur (Figure 5A). By contrast, P-WI cells that were first infected with lenti-LgT and then superinfected with lenti-p16 continued to synthesize DNA and proliferate (Figure 5B) despite high p16 expression. The presence or absence of LgT did not affect the level of p16 expressed by lenti-p16 (not shown). Similar results were obtained using HMECs. LgT prevents pre-selected HMECs from senescing when introduced prior to upregulation of p16 (Huschtscha *et al.*, 2001). However, LgT did not stimulate cell proliferation when introduced after HMECs had undergone the p16-induced senescence arrest (not shown). Finally, although 70–75% of P-WI cells expressing shRNA p16 and cultured until senescence resumed growth upon p53 inactivation by GSE-22 (Figure 4B), this was not the case when p16 was downregulated in cells that had already undergone senescence with high p16 expression. We constructed a lentivirus to express the shRNA-p16 and used it to downregulate p16 in S-WI cells. We

then superinfected the cells with lenti-GSE-22. Although GSE-22 stimulated DNA synthesis, very few cells (~1–2%) resumed proliferation (Figure 5C). Therefore, once the p16/pRB pathway is engaged, its downregulation is insufficient for DNA synthesis unless p53 is also targeted. Moreover, once the p16/pRB pathway is engaged, neither p53 nor pRB inactivation is sufficient to allow cell proliferation.

p16 suppresses the response to oncogenic RAS

Oncogenic RAS (Ha-RAS^{v12}) (Shih and Weinberg, 1982) delivers a strong mitogenic signal that transforms immortal cells (Land *et al.*, 1983). However, normal human fibroblasts respond to oncogenic RAS by a senescence growth arrest accompanied by upregulation of p16 (Serrano *et al.*, 1997). Because cells differ in their ability to upregulate p16 upon replicative senescence, we asked whether ability to upregulate p16 also influences the senescence response to mitogenic signals, such as those delivered by Ha-RAS^{v12}.

We infected S-WI and S-BJ cells with a lentivirus expressing Ha-RAS^{v12} (lenti-RAS). Lenti-RAS did not stimulate S-WI cells to initiate DNA synthesis (Figure 6A), in agreement with reports using a different fibroblast strain and plasmid microinjection (Lumpkin *et al.*, 1986). In contrast, lenti-RAS induced >20% of S-BJ cells to synthesize DNA (Figure 6B). Thus, oncogenic RAS did not stimulate DNA synthesis in senescent cells that express high p16, but modestly stimulated senescent cells that express low levels of p16.

Regardless of the p16 level, oncogenic RAS synergized with CDK4m to stimulate senescent cells to synthesize DNA (Figure 6A and B). CDK4m alone induced only 20–35% of S-WI and S-BJ cells to synthesize DNA. However, CDK4m plus RAS induced DNA synthesis in 85–90% of the cells (Figure 6A and B). Nonetheless, neither oncogenic RAS nor CDK4m, either alone or in combination, stimulated appreciable cell proliferation (Figure 6A). In S-BJ, RAS or CDK4m each induced ~20%, and the combination induced >90% of cells to undergo a few divisions. However, proliferation was limited to 2–3 PDs (see Figure 2D). Interestingly, these cells arrested growth with low p16 levels, but they were significantly higher than control p16 levels (Figure 6E), indicating that RAS can induce p16 even in cells such as BJ, which do not express p16 upon replicative senescence. Together, these results suggest that oncogenic RAS cannot sustain the growth of senescent cells, even when p16 expression is initially low.

The ability of RAS to stimulate DNA synthesis depended on the level at which it was expressed, and was abrogated by p16. We infected S-BJ cells with lenti-RAS at a 3-fold higher titer than routinely used (high RAS). High RAS did not increase the fraction of infected cells (>90% infectivity for both high and low RAS; Figure 6D), but increased the % LN of S-BJ cells from 20 to 55% (Figure 6C). This DNA synthesis was markedly suppressed by superinfection with lenti-16 (Figure 6C). Taken together, these data indicate that p16 provides a formidable barrier to reversal of the senescence growth arrest by p53 inactivation, as well as by the strong mitogenic signal delivered by oncogenic RAS.

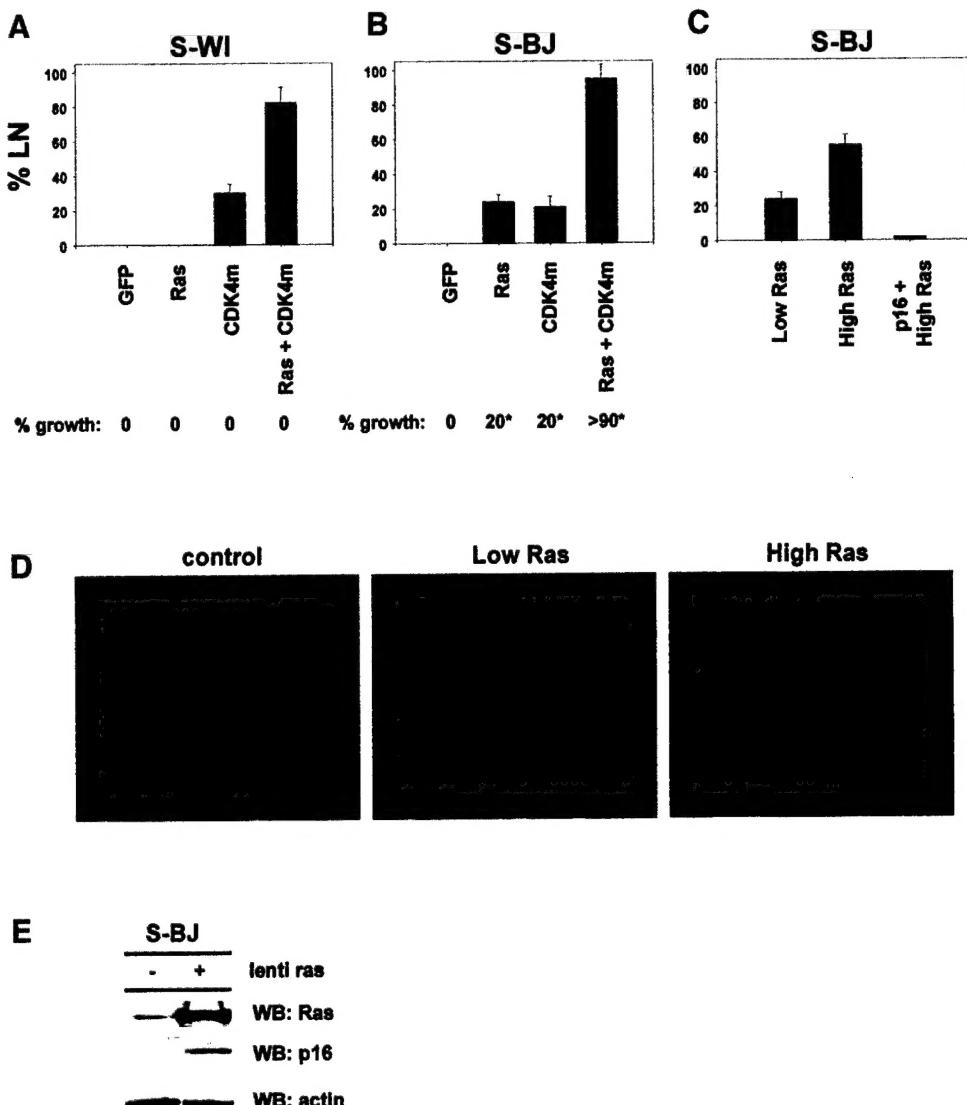


Fig. 6. Oncogenic Ras partially reverses p16-independent senescence. (A) S-WI cells do not synthesize DNA in response to oncogenic Ras. S-WI cells were infected with the indicated lentiviruses, and 72 h later assessed for ability to synthesize DNA (% LN) and proliferate (% growth), as described in Materials and methods. (B) S-BJ cells synthesize DNA and undergo limited proliferation in response to oncogenic Ras. S-BJ cells were infected with the indicated lentiviruses, and assessed for % LN and % growth. The asterisk indicates that the cells underwent limited proliferation, amounting to 3 PDs or less. (C) Mitogenic effects of Ras are concentration- and p16-dependent. S-BJ cells were infected with 1× (low Ras) or 3× (high Ras) lenti-Ras virus concentrations (determined by p24 levels, as described in Materials and methods) and % LN was measured. Where indicated, S-BJ cells were infected with lenti-p16 5 days prior to subsequent infection with high lenti-Ras. (D) Ras immunostaining. S-BJ cells were mock infected or infected with lenti-Ras at 1× or 3× virus concentrations, and immunostained for Ras. Nuclei were identified by DAPI staining.

Discussion

It is well established that the p53 and pRB pathways are critical for establishing the replicative senescence of human cells. Much less is known about the requirements for maintaining the senescence growth arrest. Our results support a model in which human fibroblasts establish and maintain the senescence growth arrest by either of two mechanisms, depending on whether p16 is expressed. Both mechanisms impose a growth arrest that cannot be reversed by known physiological signals. However, in the absence of p16 expression, the senescence arrest can be reversed by inactivation of p53. Thus, the replicative senescence of human cells is not necessarily irreversible

once established, and p16 plays a critical role in preventing its reversal by p53 inactivation (Figure 7).

In agreement with previously proposed models, our results support the idea that telomere-dependent replicative senescence depends primarily on the p53 pathway (Atadjia *et al.*, 1995; Gire and Wynford-Thomas, 1998). In some human cell strains, such as BJ, this p53-dependent growth arrest is the predominant mechanism that limits replicative lifespan, and is reversible upon inactivation of p53. Thus, we found that the replicative senescence of S-BJ cells was completely reversed by GSE-22, which inactivates p53 (Gudkov *et al.*, 1993). The arrest of these cells was also reversed by LgT and LgT-K1, which, among other activities, also inactivate p53. This reversal resulted

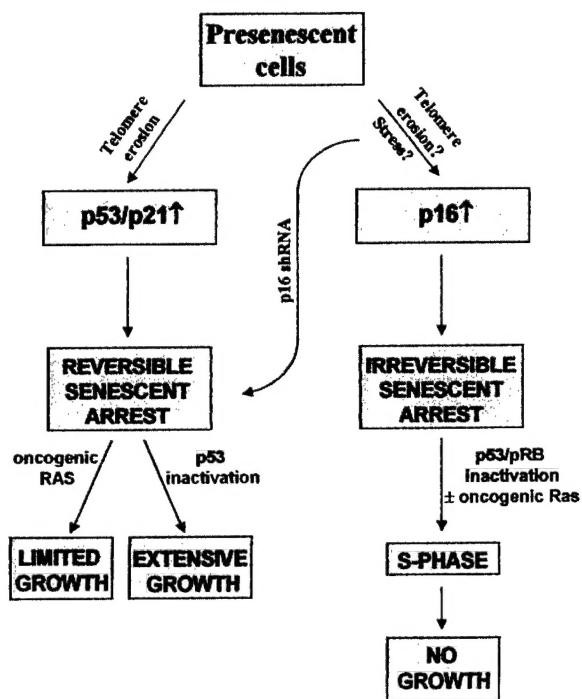


Fig. 7. Pathways leading to reversible and essentially irreversible senescence growth arrests in human cells. Proliferating cells (Presenescent) arrest growth with a senescent phenotype in response to telomere erosion, which is p53 dependent, or a combination of telomere erosion and an as yet unidentified stimulus that induces p16. The p53-dependent arrest increases p21 expression, and is reversed by p53 inactivation or oncogenic Ras. p53 inactivation results in extensive proliferation (growth) culminating in crisis, whereas Ras causes limited proliferation. Cells that senesce with high p16 can be stimulated to synthesize DNA (S-phase) upon inactivation of p53 and pRb, or pRb inactivation plus oncogenic Ras, but do not proliferate (no growth).

in extensive (>20 PDs) cell proliferation, and, eventually, the reversed S-BJ cultures ceased proliferation with characteristics of crisis.

BJ fibroblasts expressed very low levels of p16 throughout their replicative lifespan. WI-38 cells, in contrast, expressed p16 even when pre-senescent, and showed a progressive increase in expression throughout their lifespan. We suggest that BJ and WI-38 cells represent extremes in a spectrum of p16 expression in human cells, since a third fibroblast strain, 82-6, showed intermediate p16 expression. However, it remains to be seen whether the cell strains we studied here are representative among the many dozens available for study. Nonetheless, our data support the idea that the ability to induce p16 provides a second mechanism for establishing and maintaining the replicative senescence of human fibroblasts. In contrast to the block established by p53, we were unable to reverse the arrest established by p16. Direct inactivation of pRb (by LgT) or suppression of p16 expression by shRNA allowed cells with high p16 (S-WI cells, P-WI + lenti-p16) to enter the S-phase of the cell cycle upon p53 inactivation. However, these cells failed to proliferate, indicating that the p16/pRb pathway, which is known to regulate entry into S-phase, must also act subsequent to the initiation of S-phase to prevent cell division.

Of particular interest, the ability of p16 to prevent LgT-stimulated cell proliferation depended on the order of expression. Thus, LgT failed to stimulate the growth of p16-expressing cells, but ectopic p16 expression did not inhibit the proliferation of LgT-expressing cells. Similarly, inactivation of both p16 and p53 (by shRNA and GSE-22) failed to stimulate the growth of S-WI, but the same combination was very effective at reversing the growth arrest if the shRNA-p16 was expressed before WI38 cells reached replicative senescence. These findings suggest that irreversible, presumably epigenetic, changes can determine the extent to which cells are susceptible to growth stimulation by p53 inactivation. We hypothesize that once p16 is expressed, unphosphorylated pRb establishes an essentially irreversible repressive chromatin state. This repressive chromatin may then persist, even if pRb is subsequently inactivated (e.g. by LgT binding) or even if p16 itself is subsequently suppressed (for example, by shRNA) (Brehm and Kouzarides, 1999; Narita *et al.*, 2003). This model is supported by the recent finding that some senescent cells acquire heterochromatic domains that depend on pRb activity (Narita *et al.*, 2003).

In contrast to p53 inactivation, hTERT expression had no effect on the proliferation of replicatively senescent cells, regardless of p16 expression. This result indicates that cell proliferation is required for telomerase to extend the replicative lifespan or to immortalize human cells. Consistent with this idea, hTERT immortalized S-BJ cells only after they had been stimulated to proliferate by LgT or GSE-22 (data not shown). Moreover, hTERT did not alter telomere length in senescent cells, suggesting that DNA synthesis is needed for telomerase to extend the telomeres of human cells. Studies in yeast similarly suggested that an S-phase is required for telomerase to act at the telomeres (Wellinger *et al.*, 1993).

Interestingly, Ras^{v12} stimulated limited proliferation in S-BJ, but was completely inactive in S-WI, or S-BJ cells that ectopically expressed p16. Earlier microinjection studies indicated that oncogenic RAS was incapable of stimulating senescent human fibroblasts to synthesize DNA (Lumpkin *et al.*, 1986). Presumably, the cells used in this study expressed high levels of p16. Our results suggest that oncogenic RAS can overcome the growth-inhibitory effects of p53 and p21, but not p16. However, oncogenic RAS did not induce robust proliferation even in cells that express low p16, presumably because RAS itself eventually induces a senescent-like arrest (Serrano *et al.*, 1997), and, at least in BJ cells, eventually increased p16 expression. RAS also synergized with CDK4m to induce cell cycle progression, although its mechanism in this regard is not clear, given that CDK4m probably acts on both the p16/pRb and p53 pathways. Nonetheless, our results suggest that transformation by oncogenic RAS may require complete or partial inactivation of the INK4a locus.

Our results raise several important questions regarding how cells respond to senescence-inducing stimuli and the role of p16 in this response. Little is known about what determines whether, and to what extent, cells express p16. Fibroblast strains clearly differ in their propensity to upregulate p16. However, it is not clear whether this difference reflects individual-to-individual variation, or selection for spontaneous silencing of p16 in some cell

strains, as appears to be the case for HMEC. In addition, little is known about the signals that increase p16 expression. p16 induction has been proposed to be a response to the stress of standard culture conditions (Sherr and DePinho, 2000; Wright and Shay, 2000), although the nature of the culture stress is not known. One possibility is that DNA replication puts cells at risk for inaccurate re-establishment of repressive chromatin, which can result in p16 expression, and cells may differ in the efficiency with which they maintain chromatin organization.

Cellular senescence is thought to be important for preventing unregulated growth and malignant transformation in mammalian cells (Reddel, 2000; Campisi *et al.*, 2001). Moreover, the ability to undergo a senescence response may determine the efficacy of cancer therapy (Schmitt *et al.*, 2002; te Poole *et al.*, 2002). Our data indicate that p16 is crucial for ensuring the irreversibility of the senescence arrest, consistent with its important role in tumor suppression.

Materials and methods

Cells and cell culture

Human WI-38 lung, BJ foreskin and 82-6 skin fibroblasts were obtained and grown as described previously (Dimri *et al.*, 2000; Itahana *et al.*, 2001). Subconfluent cells were passaged in 20% oxygen until senescence, as determined by % LN, as described previously (Dimri *et al.*, 2000). Cultures with >75% LN were considered pre-senescent, while those with <1% LN were considered senescent. To achieve <1% LN, it was critical to maintain cultures at subconfluent densities. Senescence reversibility assays were performed on cultures that had ceased proliferation (<1% LN) for 1–3 months. Where indicated, a fixed titer (see below) of lentivirus was added simultaneously with [³H]thymidine. PDs were calculated from the cumulative cell number at each passage. When senescent cells proliferated >5–6 PDs, percentage growth was determined by clonogenic assays. When growth comprised <5–6 PDs, percentage growth was determined by counting the total number of cells per culture, and, where the senescent morphology was reversed, by counting the fraction of cells with senescent versus pre-senescent morphology per field. Clonogenic assays were performed by seeding 0.5–1 × 10³ cells per 35 mm dish and counting the number of colonies with >50 cells 14–18 days later. HMECs (M.Stampfer, Lawrence Berkeley National Laboratory) were grown as described previously (Stampfer and Bartley, 1985). In our hands, the cell strains used in this study never spontaneously gave rise to replicatively immortal variants.

Vectors and viral infections

pBABE-hTERT (Kim *et al.*, 1999) was used to produce infectious retrovirus using PT67 packaging cells (Clontech). The following DNA fragments were obtained by restriction digestion or PCR: GSE-22 (from pBabe-GSE, encoding an interfering p53 fragment) (Gudkov *et al.*, 1993), LgT and LgTK1 [from pCMV(T) and pCMV (TK1), neither of which encodes small t-antigen] (Hara *et al.*, 1996b), p16 (cDNA from E.Hara, University of Manchester), CDK4^{24C} (CDK4m; cDNA from W.Hahn, Dana-Farber Cancer Institute), EGFP cDNA (Clontech), hTERT cDNA (Counter *et al.*, 1998), Ha-RAS¹² cDNA (from pBABE-RAS) (Serrano *et al.*, 1997), and a p16 short-hairpin RNA (shRNA) expressed from the U6 promoter in MSCV-shp16 as described by Narita *et al.* (2003). DNA fragments were subcloned into the pRRL.SIN-18 lentivector, which places inserted DNA under the control of the CMV promoter (Dull *et al.*, 1998). Infectious virus was produced by transiently transfecting lentivector and packaging vectors into 293T cells as described previously (Naldini *et al.*, 1996). Viral supernatants were concentrated by ultracentrifugation, and titers determined by ELISA for p24 (viral capsid protein) using a commercial kit (Perkin-Elmer). Cells were infected with a minimum of 40 (up to 100) ng/ml p24 equivalents (1–2 ng/1000 cells) in the presence of 6 µg/ml polybrene. Test infections using lenti-GFP showed the infection efficiency was >95% using 30 ng/ml p24 equivalents. Infection efficiencies were confirmed by immunofluorescence, except for hTERT, for which antibodies suitable for immunofluorescence were not available.

Telomerase activity and telomere length determinations

Telomerase activity was determined by TRAP, using a commercial kit (Intergen, Purchase, NY), and telomere length was assessed by Southern blot analysis, as described previously (Kim *et al.*, 1999).

Western blotting and immunofluorescence

Western analysis was performed as described previously (Dimri *et al.*, 2000). Primary antibodies were p21 (BD Biosciences 556430), Ras (BD Biosciences 610001), LgT (Santa Cruz 147), p53 (Santa Cruz 6243), actin (Chemicon MAB1501), p16 (JC8, gift of J.Koh, University of Vermont) and CDK4 (NeoMarker MS-864-P1). For immunofluorescence, we seeded cells in four-well chamber slides, fixed them with 3.7% formaldehyde, permeabilized them with 0.1% Triton X-100 in phosphate-buffered saline (5 min), treated them with ice-cold methanol (20 min) and blocked with 5% goat serum (1 h). Cells were incubated with primary antibodies (2–16 h) and secondary antibodies plus DAPI (1 h) in blocking solution.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank J.Garbe for senescent HMEC, and E.Hara, W.Hahn, G.Dimri and J.Koh for valuable reagents. This work was supported by grants from the US National Institutes of Health (grant Nos AG09909 and AG16379 to to J.C. and S.W.L.), US Department of Defense (grant Nos DAMD17-00-0308 and DAMD17-01-1-0209 to to P.Y. and M.N.), California Breast Cancer Research Program (grant No. 8KB-0100 to A.K.), the Canadian Institute of Health Research to C.M.B., Jose Carreras International Leukemia Foundation to F.G. and Uehara Memorial Foundation to M.N.

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Received November 29, 2002; revised May 12, 2003;
accepted July 1, 2003